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## Hepatotoxic Cyanobacterial Blooms in Louisiana's Estuaries: Analysis of Risk to Blue Crab (*Callinectes sapidus*) Following Exposure to Microcystins

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**HEPATOTOXIC CYANOBACTERIAL BLOOMS IN LOUISIANA'S ESTUARIES:  
ANALYSIS OF RISK TO BLUE CRAB (*CALLINECTES SAPIDUS*) FOLLOWING  
EXPOSURE TO MICROCYSTINS**

A Thesis  
Submitted to the Graduate Faculty of the  
Louisiana State University and  
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requirements for the degree of  
Master of Science  
in  
The Department of Oceanography and Coastal Sciences

By  
Ana Cristina Garcia  
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## ABSTRACT

The most common toxins detected following freshwater harmful algal blooms are microcystins, a group of hepatotoxins produced by cyanobacteria (e.g., *Microcystis* and *Anabaena* spp.). Preference for filter-feeding prey, including bivalves, makes the blue crab, *Callinectes sapidus*, vulnerable to microcystin contamination and makes the commercially important crab a potential vector of microcystins. I used a combination of field and laboratory studies to determine blue crab vulnerability to microcystin contamination and consequent impacts on crab physiology.

Samples collected from a hyper-eutrophic freshwater lake, Lac des Allemands, Louisiana, were analyzed for cyanobacterial abundances and microcystins in surface water and blue crabs using light-microscopy and enzyme-linked immunosorbent assay (ELISA). Alternating blooms of *Microcystis* and *Anabaena* spp. occurred throughout the sampling period. Highest microcystins concentration in surface water ( $1.42 \mu\text{g l}^{-1}$ ) was above tolerable daily intake (TDI) guidelines set by the World Health Organization (WHO). Highest microcystin concentrations in crab tissue were 820, 65, and  $105 \mu\text{g microcystins kg}^{-1}$  in hepatopancreas, viscera, and muscle, respectively, which were close to or exceeding the WHO-TDI guidelines. The study demonstrated the ability of *Microcystis* and *Anabaena* blooms to produce toxins that accumulate in blue crab tissues and are possibly transferred to higher level consumers, including humans. Laboratory studies were aimed at understanding distributions and physiological effects of two microcystin congeners, microcystin-LR and -RR, in orally exposed blue crabs. Crabs administered 0, 10, 100, 500, or  $1000 \mu\text{g microcystin kg}^{-1}$  crab body weight<sup>-1</sup> of either congener were sacrificed 48-hours following exposure. Using ELISA, microcystins were detected in hepatopancreas and viscera, but not in muscle or hypodermis for both microcystin-LR and -RR exposed crabs. There was significant correlation between microcystin-LR uptake in

hepatopancreas and crab weight loss after 48-hours ( $P < 0.050$ ). In crabs administered daily doses of  $50 \mu\text{g}$  microcystin-RR  $\text{kg}^{-1}$  crab body weight $^{-1}$  for 7-days, significant ( $P < 0.050$ ) accumulation was seen in hepatopancreas and viscera, but not in muscle, hypodermis, or gills. Lipid hydroperoxides in hepatopancreas were significantly ( $P < 0.050$ ) altered following exposure, indicating oxidative stress. These studies indicate that natural populations of blue crabs may be subjected to physiological stress following microcystin accumulation.

# CHAPTER 1

## INTRODUCTION TO HEPATOTOXIC CYANOBACTERIA AND BLUE CRAB FISHERIES IN LOUISIANA

### 1.1 General Introduction

The dynamic water movements within estuaries produce ecosystems that are unrivaled in terms of productivity. Estuaries provide critical nursery grounds, and many of the organisms that depend on these highly productive areas support important commercial fisheries. Primary producers (including algal resources) support the numerous species of zooplankton, planktivorous/herbivorous fishes, and filter-feeding bivalves, which in turn support large predatory fishes and crustaceans.

One such estuary system is the Barataria-Terrebonne Estuary System of southern Louisiana. This estuary system covers an area of approximately 16,835 km<sup>2</sup> and contains 19 major lakes (McKenzie et al., 1995), including Lac des Allemands, a freshwater lake in the uppermost reaches of the estuary. The lake is well known for its production of catfish, including *Ictalurus furcatus* (blue catfish) and *I. punctatus* (channel catfish), and blue crab, *Callinectes sapidus*. Lake Pontchartrain, an oligohaline lagoonal estuary adjacent to the city of New Orleans, is also known for its high production of Louisiana blue crab. However, natural and anthropogenic factors (e.g., subsidence, cultural eutrophication, industrial contamination, levee construction, and sea-level rise) are endangering the future of these systems.

Anthropogenic nutrient enrichment of estuaries can have a direct impact on phytoplankton primary production and community composition. Harmful algal blooms (HABs) occur when phytoplankton accumulate to levels producing negative impacts due to abundance, morphology, or phycotoxin production. Most toxin producing phytoplankton can be classified as either dinoflagellates or cyanobacteria (Glibert et al., 2005), and the most commonly reported

phycotoxins from fresh or brackish waters are a group of cyanobacterial hepatotoxins known as microcystins (Sivonen, 1996; Sivonen and Jones, 1999; Chorus and Bartram, 1999; Ibelings et al., 2005). Among this group containing more than 60 structural variants (Codd et al., 1999), most studies focus on microcystin-LR, and to a lesser extent, microcystin-RR as these congeners are among the most common microcystins occurring in natural blooms (Ibelings and Havens, 2007).

Microcystins have been compared in toxicity to chemical organophosphate nerve agents (Dawson, 1998) and have caused death in aquatic and terrestrial organisms (Carmichael, 1994, 1997). Human deaths through exposure during hemodialysis have also occurred (Carmichael et al., 2001; Azevedo et al., 2002), and microcystins have been implicated as promoters of primary liver cancer (Ueno et al., 1996; Ito et al., 1997). Recently, Chen et al. (2009) detected microcystins in serum samples taken from fishermen from Lake Chaohu, China, where dense blooms of cyanobacteria regularly occur. Symptoms of microcystin intoxication in humans include diarrhea, vomiting, piloerection, weakness, pallor, and labored breathing (Dawson, 1998; Codd et al., 1999), making diagnosis of microcystins related illnesses difficult due to similarity of symptoms with those commonly associated with other food-borne illnesses. Toxicity of microcystins occurs via uptake into hepatocytes, leading to irreversible inhibition of serine/threonine protein phosphatases PP1 and PP2A. The ensuing imbalances in protein phosphorylation cause disruption of the cytoskeleton of the liver and hepatic hemorrhage (Falconer et al., 1981; MacKintosh et al., 1990). However, oxidative stress has also been implicated as a toxicological consequence to microcystins exposure. Oxidative stress is the disruption in prooxidant/antioxidant balance in favor of increased prooxidants or as the disruption of redox signaling and control (Amado and Monserrat, 2010). In several recent studies, microcystins uptake has been related to increased production of reactive oxygen species



(ROS), leading to increased lipid peroxidation, DNA damage, mitochondrial damage, and stimulation of antioxidant defense systems (Amado and Monserrat, 2010 and references therein). Microcystins are highly stable and resistant to boiling (Falconer and Humpage, 2005; Chen et al., 2008), and most natural reductions of microcystins occur due to dilution and biodegradation (Cousins et al., 1996; Chen et al., 2008 and references therein).

In this introduction, I first provide information from the literature on the trophic transfer of microcystins in aquatic food webs. Then I briefly discuss the commercial importance of blue crab, *Callinectes sapidus*, fisheries and blue crab vulnerability to phycotoxin contamination. Finally, I provide brief descriptions of the research chapters that follow.

### **1.1.1 Microcystin Accumulation and Trophic Transfer in Aquatic Food Webs**

Microcystins are water-soluble, polar compounds with high molecular weights ( $\sim 1000 \text{ g mol}^{-1}$ ). When found in the dissolved state (from lysis of cyanobacterial cells), microcystins are not readily accumulated due to their inefficiency in passing through lipid bilayers (Ibelings and Havens, 2007; Amado and Monserrat, 2010). Thus, most exposure to microcystins occurs via food webs. Microcystins have been found to accumulate in tissues of aquatic organisms occupying various feeding guilds (i.e., phytoplanktivorous, omnivorous, and carnivorous organisms), indicating trophic transfer of these toxins in natural ecosystems (Ibelings et al., 2005). However, monitoring data from a lake food web in IJsselmeer, The Netherlands indicated that no biomagnification of microcystins occurs, since concentrations found in filter feeders were always lower than those in phytoplankton (Ibelings et al., 2005). Although, feeding strategy of aquatic organisms appears to be the main determinant of toxin exposure, meta-analysis of multiple studies indicated that species most frequently exposed to cyanobacterial toxins (e.g., bivalves) may also have among the highest physiological tolerances and depuration rates (Sipia et al., 2002; Pereira et al., 2004; Kankaanpää et al., 2005; Ibelings and Havens, 2007). Expulsion

of microcystins via pseudo-feces may serve as the primary defense mechanism in these sessile organisms (Juhel et al., 2006; Armado and Monserrat, 2010). However, the accumulation of microcystins in other trophic compartments is more variable. For example, the microcystin accumulation and consequent toxicity in different fishes is thought to vary due to differences between the surface areas and pHs of gastro-intestinal (GI) tracts of carnivorous and herbivorous fishes (Ibelings and Havens, 2007 and references therein).

### **1.1.2 Blue Crab Fisheries**

According to the Louisiana Department of Wildlife and Fisheries (LDWF), blue crab ranked seventh among all commercially harvested seafood species in the United States, fifth among commercially harvested seafood species in the Gulf of Mexico, and fourth among commercially harvested seafood in Louisiana in 2002 according to dockside value (LDWF, 2004). Only commercial values for white shrimp, menhaden, and brown shrimp, exceeded the value of Louisiana's blue crab fishery in 2002 (LDWF, 2004).

Louisiana is the largest producer of blue crabs (also for shrimp and oyster production) in the United States, with approximately 26% of the nation's blue crab production occurring in Louisiana in 2001 (Lester et al., 2005). A majority of Louisiana's blue crab harvest occurs from the 13 parishes within the Barataria-Terrebonne Estuary System (McKenzie et al., 1995). In 2006, Louisiana commercial blue crab landings totaled approximately 52.4 million pounds (23.8 million kg) and supported 3289 Louisiana residents in related jobs. However, Louisiana commercial blue crab landings decreased 16% to approximately 43.9 million pounds (19.9 million kg) and had a dockside value of approximately \$44.8 million in 2007. This decrease was likely due to increased foreign imports of crabmeat, lowering domestic prices (Fig. 1.1) (LDWF, 2008). The demersal blue crab largely preys on sessile filter-feeding clams, mussels, and oysters, making them vulnerable to phycotoxin contamination. Among the dominant prey items for

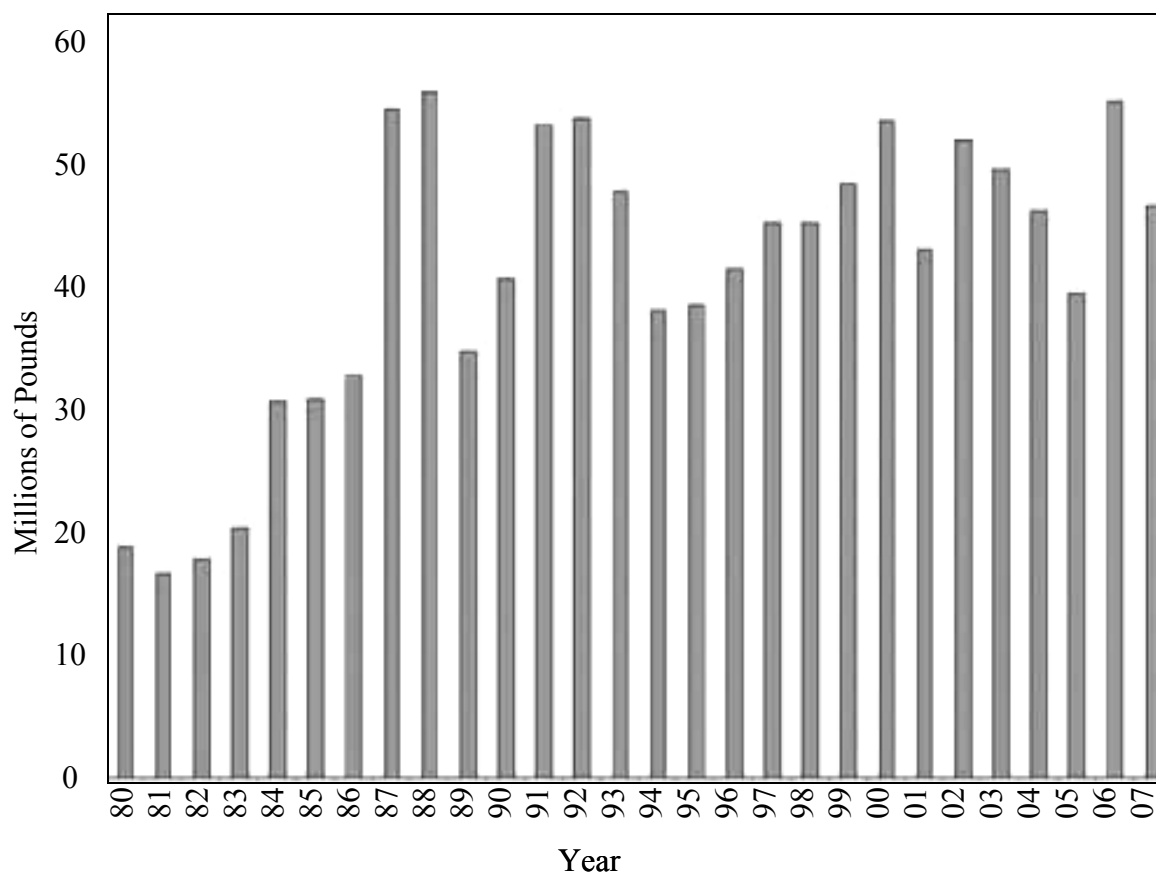


Figure 1.1. Louisiana commercial blue crab, *Callinectes sapidus*, landings (millions of pounds) from 1980 until 2007 (modified from LDWF, 2008).

Louisiana blue crab is *Rangia cuneata* (Darnell, 1958), a non-selective filter-feeding freshwater clam. *R. cuneata* are abundant in the upper freshwater portions of the Barataria-Terrebonne Estuary System, overlapping with distributions of major blue crab commercial fisheries. Blue crabs are opportunistic feeders and are commonly cannibalistic, carnivorous, omnivorous, and/or detritivorous. Blue crab feeding strategies and close association with sediments make chironomid larvae living within sediments (Galván et al., unpublished data) and bivalve pseudofeces other potential routes for exposure to phycotoxins.

## **1.2 Chapter Introductions**

In chapter 2, I describe cyanobacterial communities occurring in the upper freshwater portions of the eutrophic Barataria Estuary System of Louisiana and examine corresponding

microcystin concentrations in particulate water samples. Cyanobacteria species enumerated include toxic *Cylindrospermopsis* cf. *raciborskii*, *Raphidiopsis* cf. *curvata*, *Anabaena* cf. *flos-aquae*, *Anabaena* cf. *circinalis*, *Microcystis* cf. *aeruginosa*, and non-toxic *Merismopedia* species. I describe microcystin accumulation for the first time in tissues (hepatopancreas, viscera, and muscle) of the key-predator blue crab, *Callinectes sapidus*. Provisional daily intake guidelines set by the World Health Organization (WHO, 1998) are applied to assess risk associated with consumption of microcystin contaminated water and blue crab tissues.

In chapter 3, I investigate the accumulation of purified microcystin-LR and -RR in tissues (hepatopancreas, viscera, muscle, hypodermis, and gills) of orally exposed blue crabs. Crabs were either exposed for 48-hours to a single dose ranging from 0 – 1000  $\mu\text{g kg}^{-1}$  crab body weight<sup>-1</sup> or to daily doses of 50  $\mu\text{g microcystin-RR kg}^{-1}$  crab body weight<sup>-1</sup> for 7 days (168 hours). Lipid hydroperoxides, biomarkers for oxidative stress, are compared between the treatment groups.

In chapter 4, I summarize the findings from chapters 2 and 3 and indicate how this work adds to the current understanding of microcystin contamination in aquatic food webs. I further evaluate the consequences of microcystin intoxication in blue crab and additional commercial fisheries in Louisiana.

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## CHAPTER 2

### EVALUATING THE POTENTIAL RISK OF MICROCYSTINS TO BLUE CRAB (*CALLINECTES SAPIDUS*) FISHERIES AND HUMAN HEALTH IN AN EUTROPHIC ESTUARY\*

#### **2.1 Introduction**

Algal blooms are increasing worldwide due to eutrophication of aquatic environments (Anderson et al., 2002). Anthropogenic nutrient enrichment of rivers and estuaries can have a direct impact on algal species composition and the formation of noxious and toxic blooms and surface scums. The Barataria-Terrebonne Estuarine System (BTES) of Louisiana is an example of a eutrophied system, and toxic and noxious phytoplankton blooms may be increasing as a consequence of excess nutrients (Rabalais et al., 1995).

Although many algal classes including diatoms are capable of producing toxins, most toxic phytoplankton can be classified as either dinoflagellates or cyanobacteria (Glibert et al., 2005). Some cyanobacteria are of particular concern due to their production of a broad assortment of toxic metabolites. Species in the *Microcystis*, *Anabaena*, *Cylindrospermopsis*, and *Raphidiopsis* genera are capable of producing multiple toxins, with some cyanobacterial toxins having as many as 60 known structural variants (e.g., microcystins) (Codd et al., 1999).

Cyanotoxins in contaminated drinking- and recreational-water and food sources can pose a serious hazard to both wild and domestic animals, and to humans alike. Cyanobacterial toxins are classified, based on their modes of toxicity, as: hepatotoxins (chemicals causing liver injury), neurotoxins, cytotoxins, dermatoxins, and irritant toxins (Codd et al., 1999; Sotero-Santos et al., 2008). The most common cyanobacterial toxins found in blooms from fresh and brackish waters

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\* Reprinted by permission of the journal *Harmful Algae*: Garcia, AC, Bargu, S, Dash, P, Rabalais, NN, Sutor, M, Morrison, W, Walker, ND. Evaluating the potential risk of microcystins to blue crab (*Callinectes sapidus*) fisheries and human health in a eutrophic estuary. *Harmful Algae* 9 (2010) 134–143.



are cyclic peptides known as microcystins (Sivonen, 1996; Sivonen and Jones, 1999). These toxins are highly water-stable and resistant to boiling, and thus pose a threat to water and food quality if not properly monitored (Falconer and Humpage, 2005). Microcystins are produced by several genera including: *Microcystis*, *Planktothrix* (*Oscillatoria*), *Anabaena*, *Nostoc*, *Anabaenopsis*, and *Hapalosiphon* (Carmichael et al., 2001). The hepatotoxicity of microcystins depends on their degree of inhibition of protein phosphatases (MacKintosh et al., 1990), leading to symptoms of weakness, cold extremities, labored breathing, vomiting and diarrhea, possibly causing death due to liver hemorrhaging and respiratory arrest (Codd et al., 1999). Long-term exposure to sub-lethal concentrations of microcystins has been implicated in the promotion of tumors (Ito et al., 1997), making prolonged blooms of microcystin-producing cyanobacteria perilous.

The upper Barataria estuary receives multiple nutrient inputs from runoff of lake-shore development, intense agriculture, and unsewered areas, as well as diverted waters from the Mississippi River. These factors, in combination with other issues of residence time, temperature, food web dynamics, concentrations of micronutrients, and quantity and relative abundances of major nutrients supplied into the estuary, may have significant impacts on phytoplankton species composition, frequency and intensity of harmful algal blooms, and toxin production (Anderson et al., 2002, 2008 and references therein). Blooms of *Anabaena*, *Microcystis*, *Cylindrospermopsis*, *Raphidiopsis*, and *Aphanizomenon* species occur in the fresh and brackish waters of the upper Barataria estuary, and nutrient additions, primarily nitrogen, in bioassay microcosms with water from this area stimulated the growth of these cyanobacteria (Ren et al., 2008, 2009).

Few cases of cyanobacterial toxin poisonings have been documented in humans, but this may be due to a lack of information regarding vectors, inability to identify and link symptoms,

and inadequate methods of toxin detection (Carmichael et al., 2001). Intoxication of aquatic organisms following exposure to microcystins is better documented, especially in freshwater fish and other pelagic organisms (see Magalhães et al., 2001; Mohamed et al., 2003; Simoni et al., 2004; Xie et al., 2005). However, exposure to microcystins has also been shown to adversely impact benthic and burrowing organisms, including bivalves, crayfish, and crabs (Amorim and Vasconcelos, 1999; Vasconcelos et al., 2001; Simoni et al., 2004; Dewes et al., 2006; Chen and Xie, 2007). Studies to describe benthic or demersal vectors of these toxins, however, are very limited.

The blue crab, *Callinectes sapidus*, is an opportunistic feeder known to be cannibalistic, omnivorous, and/or a carrion feeder. Blue crabs congregate on clam, mussel, and oyster beds (Hughes and Seed, 1981; Kennedy and Cronin, 2006), making them strong candidates for phycotoxin contamination via these filter feeders. Among the dominant prey items for blue crabs is the non-selective, filter-feeding freshwater clam, *Rangia cuneata* (Darnell, 1958). *R. cuneata* is abundant in the upper BTES (personal communication, K. Galván) and may represent an important link between cyanotoxins and blue crabs within this system. The area is also well known for its production of several species of demersal catfish, including *Ictalurus furcatus* (blue catfish) and *I. punctatus* (channel catfish). Deceased fish, often associated with intense algal blooms and low dissolved oxygen concentrations, could also serve as a potential food source for the carrion-feeding blue crab. To date there has been no study evaluating microcystin concentrations in the edible estuarine crab species, *C. sapidus*, a widely consumed demersal species.

Louisiana is the leader in production of blue crabs in the United States, with a majority of them being harvested from the 13 parishes within the BTES (McKenzie et al., 1995). The

close proximity of the edible blue crab with their filter-feeding prey in this region, may establish a dietary link between cyanotoxin-producing algae and human consumers of blue crab.

The aims of this study were to analyze the efficiency of current microcystin extraction methods, document the presence and abundance of toxic cyanobacteria in the freshwater lake, Lac des Allemands in southeastern Louisiana, and assess microcystin concentrations in surface water and blue crabs taken from Lac des Allemands. The data collected in this study were further used to assess potential risk to higher trophic level organisms, including humans.

## **2.2 Materials and Methods**

### **2.2.1 Description of the Study Site**

Lac des Allemands (29° 55' 58.1" N, 90° 34' 27.96" W) is a 49 km<sup>2</sup> freshwater lake (salinity <1 psu) in the uppermost part of the Barataria estuary in southeastern Louisiana (Fig. 2.1). The trophic status of the lake (Carlson, 1977) is hyper-eutrophic with chlorophyll *a* (Chl *a*) biomass levels of 40 µg l<sup>-1</sup> to 185 µg l<sup>-1</sup> (Ren et al., 2008, 2009). Nutrient inputs into Lac des Allemands primarily result from discharges of wastewater treatment plants, agricultural runoff, and storm water pumps (Rabalais et al., 1995). Figure 2.1 shows Vacherie Canal, Bayou Lassene, Bayou Boeuf and Bayou Chevreuil in relation to Lac des Allemands. Vacherie Canal, which has a series of unsewered fishing camps at its terminus with Lac des Allemands, Bayou Lassene, and Bayou Boeuf all drain extensive sugar cane fields to the northwest and southwest. Following rain storms, Bayou Chevreuil becomes turbid with runoff from fertilizer plants along the Mississippi River to the west, contributing to the hyper-eutrophic status of this lake.

### **2.2.2 Sampling and Water Quality Data**

*In situ* water quality data for temperature and salinity were obtained at 30-min intervals using a YSI (Yellow Springs Instrument) Model 6600 sonde deployed (29° 55' 6.78" N, 90° 33' 42.00" W) to 0.3 m below the water surface in Lac des Allemands. The YSI sonde was checked

for fouling during each sampling trip, and was switched out once for calibration. YSI data were checked for anomalous values upon the conclusion of each sampling trip. Surface whole water samples (2 liters) were collected in clean Nalgene bottles monthly between December 2006 and February 2007, biweekly during March 2007, and weekly between April and June 2007 from four stations (Table 2.1) within Lac Des Allemands (Fig. 2.1). Coordinates and depths of all sampling sites are summarized in Table 2.1. Subsamples for the determination of Chl *a* and microcystin concentrations were transferred on ice to the laboratory. For the determination of Chl *a* concentrations, replicate 100 ml aliquots of surface water were filtered (<50 kPa) onto 4.7 cm diameter glass fiber filters (Whatman GF/F), immediately frozen, and stored at -80 °C. The filter papers were shipped overnight on dry ice to Dr. James L. Pinckney at the Department of Biological Sciences, University of South Carolina, Columbia, for High Performance Liquid Chromatography (HPLC) analysis (Pinckney et al., 1996). Subsamples (100 – 400 ml aliquots of surface water) for determination of particulate microcystin concentrations were collected between April and July 2007, filtered (<40 kPa) onto 2.5 cm diameter glass fiber filters (Whatman GF/F), and kept frozen (-20°C) until analysis. Subsamples (100 ml) were also collected for species composition and abundance of cyanobacteria, preserved with 2% glutaraldehyde and kept in a dark and cool location until analysis. Adult blue crabs (100-196 mm carapace width;  $n=35$ ; Table 2.2) were collected weekly from the same four sites, corresponding to where surface whole water samples were collected (Fig. 2.1). Crabs were collected during the commercial and recreational crab harvesting seasons between June and July 2007 (Table 2.2) using wire crab traps, baited with discarded heads of farm-raised catfish, which were set at the bottom of each sampling site (Table 2.1). Traps were removed after 1-2 hours in the water in order to prevent unnecessary bait contamination in viscera samples. Crabs were sacrificed by

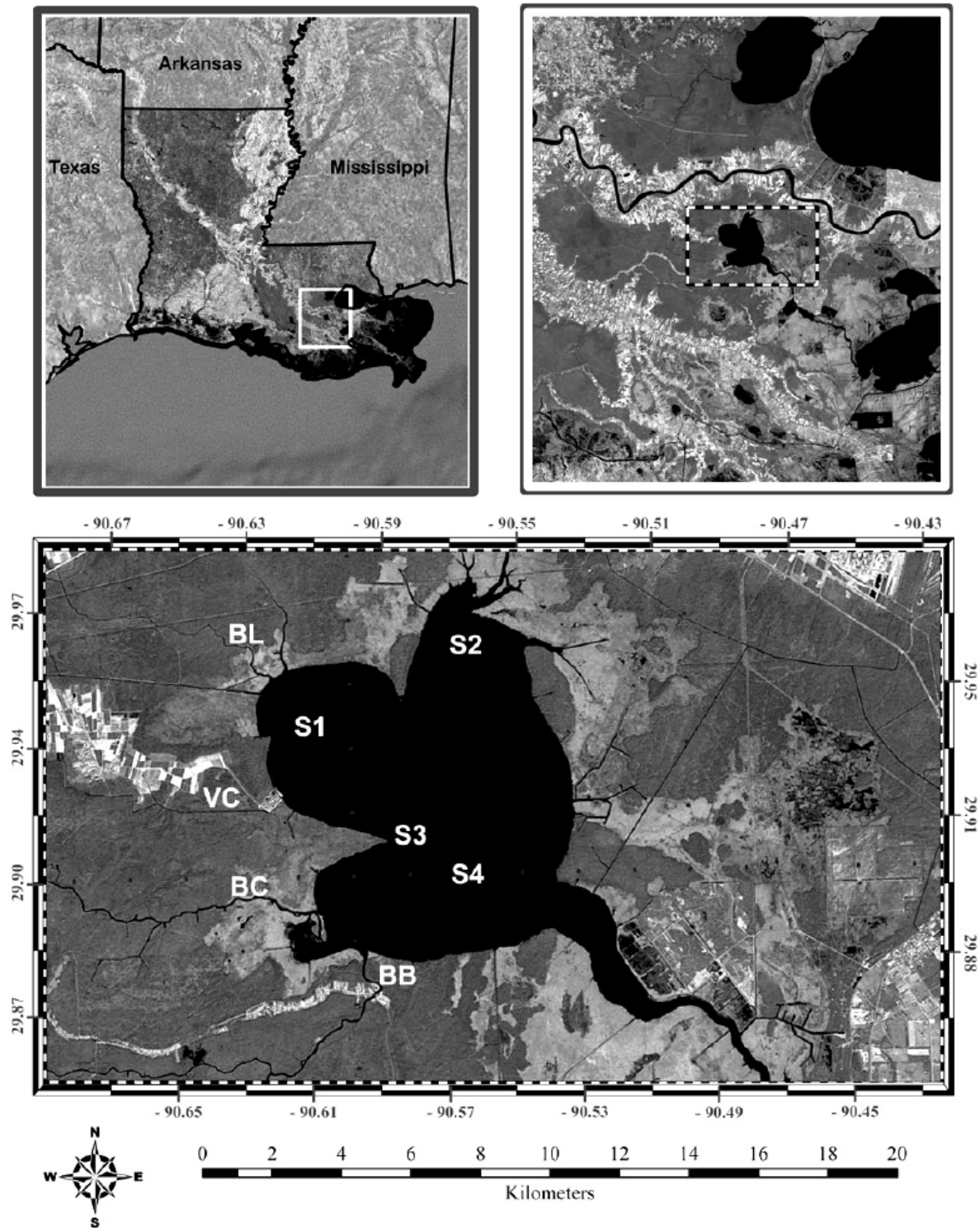


Figure 2.1. Location of Lac des Allemands within the upper Barataria estuary, with four sample sites indicated. Points of significant nutrient loading are BB-Bayou Boeuf, BC-Bayou Chevreuil, BL-Bayou Lassene, VC-Vacherie Canal.

length (measured at the widest point on the carapace including the lateral spines) and wet body

placement in an ice bath and were kept frozen (-20°C) until dissection. Before dissection, the carapace weight were recorded (Table 2.2). Carapace was removed by making an incision at the abdominal segment (adjacent to the swimming leg) then cutting across the carapace edge towards the lateral spines. We then cut across the anterolateral teeth until carapace and epidermis could be removed cleanly. Hepatopancreas (including anterior, lateral, and posterior portions), viscera (including stomach and intestines terminating at the anus), and muscle (between the visceral cavity and leg joints and from the claws) tissue, were dissected, and weighed (tissue wet weight, data not shown), and immediately extracted for toxin analysis. Additional viscera were dissected from crabs ( $n=8$ ) collected opportunistically from S2, S3, and S4 within Lac des Allemands in July 2007. These samples were further dissected to separate gut contents from

Table 2.1. Location and depth (m) of four sampling sites for surface water and blue crabs.

Site	Latitude	Longitude	Depth (m)
Site 1	29° 56' 34.66" N	90° 36' 50.27" W	1.87
Site 2	29° 56' 34.66" N	90° 34' 03.19" W	2.00
Site 3	29° 55' 09.47" N	90° 35' 00.08" W	2.20
Site 4	29° 54' 23.68" N	90° 34' 07.26" W	2.31

Table 2.2. Sampling date, location, carapace width (CW) (mm) range, wet body weight (g) range (in carapace), and number of blue crabs caught ( $n=35$  total) between June and July 2007. Asterisks (\*) indicate when tissue samples from multiple crabs were pooled for analysis.

Date	Sampling Site				Blue Crab	
	Site 1	Site 2	Site 3	Site 4	CW Range (mm)	Weight Range (g)
6/15/2007	NC	$n=1$	NC	NC	115	103
6/21/2007	$n=1$	NC	NC	NC	120	105
6/29/2007	NC	NC	$n=1$	$n=1$	155-160	139-156
7/5/2007	NC	NC	$n=3^*$	NC	150-153	144-170
7/9/2007	NC	$n=5^*$	NC	$n=1$	100-153	114-155
7/11/2007	$n=3$	NC	NC	NC	178-195	254-262
7/18/2007	$n=1$	$n=2$	$n=4^*$	$n=2^*$	105-174	104-174
7/26/2007	$n=1$	$n=4^*$	$n=5^*$	NC	135-196	100-261

$n$  represents number of crabs captured

NC, no crabs caught

CW, carapace width including spines

stomach and intestinal lining, preserved with 4% buffered formalin, and kept refrigerated until gut content analysis.

### **2.2.3 Enumeration of Cyanobacteria**

The entire contents of a Sedgwick-Rafter (1.0 ml) chamber, or a minimum of 200 cells, were counted on a compound microscope to determine abundance of *Anabaena* spp. (Venrick, 1978). Determining cell abundances for colonial *Microcystis* spp. is difficult, and cell counts are often highly variable due to irregular shape and size of mucilaginous colonies. Sonication treatment and other methods aimed at dispersing cells from colonies are often applied to monocultures of *Microcystis* to resolve these issues (Joung et al., 2006). These methods, however, cannot be applied to samples containing mixed phytoplankton communities due to similarities between *Microcystis* cells separated from colonies, and those cells belonging to other species (e.g., *Merismopedia* spp.). Colonies of mucilaginous *Microcystis* and all other cyanobacteria genera were enumerated using an imaging microscope (Fluid Imaging Technologies (Sieracki et al., 1998)), and particle properties were obtained using Visual Spreadsheet software (Fluid Imaging Technologies) in order to compare percent relative abundances between genera. Area based diameter (ABD) was measured for particles in a 1.0 ml sample. ABD is the diameter of a solid circle if all of the pixels deemed to be part of a single particle were pushed into the circle, and was used for calculation of mean colony or chain surface area. Total surface area was obtained by multiplying the mean surface area by the number of colonies or chains. Comparing the total surface area for each genus to the combined total surface areas for all cyanobacteria present allowed us to infer relative abundances for each genus without the need to disperse cells from colonies. *Raphidiopsis* and *Cylindrospermopsis* spp. were treated as a single group because we could not distinguish the morphologically similar

genera using the imaging microscope. Trends in relative abundances of cyanobacteria observed from the imaging microscope were verified using a compound microscope.

#### **2.2.4 Microcystin Extraction from Cyanobacteria**

A variety of techniques for lysing cyanobacterial cells include microwaving, sonicating, shaking, and/or freezing and thawing (Sangolkar et al., 2006). To date, no standard cyanotoxin extraction method exists, and little is known about the efficiency of these mechanical lysing methods. Several commonly used mechanical cell lysing techniques for cyanobacteria were evaluated using a common extraction solution, methanol: water: acetic acid (50:49:1 v/v, Boyer, 2008) (Table 2.3). Highest extraction efficiency (93.3%) of particulate material retained on filters was achieved following a modified protocol from Boyer (2008), and subsequent extractions were performed at room temperature using this method. Extraction solution (5 ml) was added to filters, which were vortexed (1 minute) to displace cells from the filter, sonicated (2 minutes, 30-40 W) in an ice bath using a sonicator probe, and centrifuged (10 minutes, 3000 rpm) to remove cellular debris. The supernatant was removed and filtered through a 0.2  $\mu$ m syringe filter (Corning). The remaining pellet was re-suspended using 5 ml of extraction solution before repeating the process and finally pooling the filtered supernatant.

#### **2.5 Microcystin Extraction from Blue Crab Tissues**

Microcystin extraction was performed on hepatopancreas, viscera, and muscle of blue crab. When necessary to obtain sufficient blue crab tissue for analysis or when appropriate, samples collected from a single crab trap on the same date were pooled (Table 2.2). Pooled tissue samples or tissue dissected from a single crab were homogenized separately for each tissue type (hepatopancreas, viscera, and muscle) using a hand-held tissue homogenizer. A 4 g aliquot of homogenized tissue was then collected and extracted using 1:4 ratio of tissue to 75% methanol, sonicated (2 minutes, 30-40 W), and centrifuged (20 minutes, 3500 rpm) (modified from Krienitz



Table 2.3. Evaluation of common cell lysing methods for cyanobacterial cells using extraction solvent (methanol: water: acetic acid (50:49:1)) as described by Boyer 2008.

<b>Mechanical Cell Lysing Methods in Reference</b>	<b>Reference</b>	<b>Modified Procedure</b>	<b>% Recovery</b>
Ultrasonication	Boyer 2008	5 ml of extraction solvent added to filters, vortexed (1 minute), sonicated (2 minutes, 30-40 W) on ice, centrifuged (10 minutes, 3000 rpm), pellet re-suspended using 5 ml of extraction solvent, repeat previous process pooling filtered supernatant	93.3%
Boiling	Metcalf and Codd, 2000	Filters boiled for 5 minutes in 10 ml of extraction solvent added to filters, vortexed (1 minute), sonicated (2 minutes, 30-40 W) on ice, centrifuged (10 minutes, 3000 rpm), supernatant collected and filtered	66.7%
Freeze/Thaw	Ortea et al., 2004; Akcaalan et al., 2006	Filters were frozen and thawed for 3 cycles, 10 ml of extraction solvent added to filters, vortexed (1 minute), sonicated (2 minutes, 30-40W) on ice, centrifuged (10 minutes, 3000 rpm), supernatant collected and filtered	53.3%
Continuous Stirring Overnight	Moollan et al., 1996; Mohamed et al., 2003	10 ml of extraction solvent added to filters, covered and stirred continuously overnight in ice bath, vortexed (1 minute), sonicated (2 minutes, 30-40 W) on ice, centrifuged (10 minutes, 3000 rpm), supernatant collected and filtered	40.0%

et al., 2003). Supernatant was collected, filtered through 0.2  $\mu\text{m}$  syringe filter (Corning), and stored at  $-20^{\circ}\text{C}$  until analysis by immunoassay. The microcystin extraction efficiencies were tested for different blue crab tissues by performing spike-recovery experiments on blue crabs purchased from a local seafood market. Subsamples of dissected tissues from these crabs were homogenized, injected with  $0.75\ \mu\text{g}\ \text{l}^{-1}$  microcystin-LR (Sigma Chemical Company, USA), and extracted as described above. Control subsamples, not injected with the toxin, were also analyzed for presence of microcystins. Microcystin concentrations that were detected in control subsamples were subtracted from the recovery concentration measured in spiked subsamples. Extraction efficiency in hepatopancreas, viscera, and muscle was found to be 74.5%, 98.3%, and 50%, respectively.

#### **2.2.6 Toxin Analysis**

Microcystin (MC) equivalents in surface water subsamples and blue crab tissues were determined using a commercially available highly sensitive ( $0.04\ \mu\text{g}\ \text{MC}\ \text{l}^{-1}$ ) enzyme-linked immunosorbent assay (ELISA) kit (Abraxis LLC), with a detection range of 0.05 to  $5.0\ \mu\text{g}\ \text{MC}\ \text{l}^{-1}$ . The indirect competitive assay allows for the congener-independent detection of microcystins, without cross-reacting with non-related algal compounds. Samples were analyzed following the protocol included in the kit, with each sample run in duplicate and at several dilutions in order to reduce interference from matrix effects (impact of physical components of the sample on measurement of the analyte). Absorbance was read at 450 nm using a microplate ELISA photometer.

#### **2.2.7 Crab Gut Content Analysis**

Analysis for bivalve shell fragments may not estimate the totality of bivalve consumption by blue crabs, as some individuals may pry open these prey and consume only soft tissues, rendering them unidentifiable in gut contents. Therefore, blue crab gut contents were examined

only to qualitatively assess consumption of filter-feeding bivalves, thereby establishing a potential oral route for toxin uptake. Samples were diluted with DI water and analyzed for presence of bivalve shell fragments using a dissecting microscope. General observations of prey items commonly found in blue crab stomachs were also made.

### 2.2.8 Assessment of Human Risk Associated with Crab Consumption

Estimated daily intake (EDI) calculations, included in the discussion, are aimed at assessing the potential exposure of humans to toxins and are necessary before comparing measured concentrations of microcystins in tissue samples to the World Health Organization (WHO) tolerable daily intake (TDI) provisional guidelines. Assuming mass ratios based on measurements of hepatopancreas, viscera, and muscle tissue of 2:1:20 and that each crab yields a total of 50 g (approximately 10% edible meat yield by weight (Gates and Parker, 1992)), we can calculate EDI as a function of human body weight and the number of whole crabs consumed per day, using the following formula:

$$EDI \text{ (general)} = \frac{\left(\frac{2}{23}\right)[\mu\text{g MC kg}^{-1} \text{HP}] + \left(\frac{1}{23}\right)[\mu\text{g MC kg}^{-1} \text{Visc}] + \left(\frac{20}{23}\right)[\mu\text{g MC kg}^{-1} \text{Musc}]}{\text{Body Mass (kg)}} \times 0.050 \text{ kg} * n, \quad (1)$$

where microcystins are abbreviated as MC, hepatopancreas as HP, viscera as Visc, muscle as Musc, and  $n$  represents the number of crabs from which all edible tissues are consumed.

Based on personal communication with multiple Louisiana seafood distributors, we found the usual serving size for boiled blue crab ranges from 4 to 8 crabs, depending on the size of the crab. Using this estimation, we can examine a more specific case, where a 60 kg human adult eats selected tissue from 6 blue crabs (300 g) per day. If this adult were to consume only hepatopancreas or only muscle tissue from the 6 crabs, we use the following equations:

$$EDI \text{ (HP)} = \frac{\left[\mu\text{g MC kg}^{-1} \text{HP}\right]}{60 \text{ kg Body Mass}} \times 0.026 \text{ kg HP tissue}, \quad (2)$$

or

$$EDI (Musc) = \frac{[\mu g MC kg^{-1} Musc]}{60 kg Body Mass} \times 0.26 kg Musc tissue. \quad (3)$$

We focus on EDI calculations for hepatopancreas and muscle tissue only, as the viscera is generally not sought after in peeled crab, and is therefore not considered an important dietary source of microcystins. However, inclusion of the viscera tissue is considered important when all edible portions of blue crab are consumed, as often occurs with soft shelled crab (personal communication, multiple Louisiana seafood restaurants). We can then calculate the EDI for a 60 kg adult when hepatopancreas, viscera, and muscle tissue from six whole crabs (300 g of edible tissue, as previously assumed) are consumed per day, using the following formula:

$$EDI = \frac{\left(\frac{2}{23}\right)[\mu g MC kg^{-1} HP] + \left(\frac{1}{23}\right)[\mu g MC kg^{-1} Visc] + \left(\frac{20}{23}\right)[\mu g MC kg^{-1} Musc]}{60 kg Body Mass} \times 0.3 kg. \quad (4)$$

If we did not measure the toxicity in any one of the tissue types (hepatopancreas, viscera, or muscle) for any given sample, then for the purposes of calculating whole blue crab EDI, we assumed the toxicity to be zero in that particular tissue sample and accepted the potentially underestimated values.

## **2.3 Results**

### **2.3.1 Chlorophyll *a* and Phytoplankton Community Structure**

Chlorophyll *a* (Chl *a*) concentration ranged from 1.89 to 208  $\mu g l^{-1}$ . The mean concentration for the entire 8 months of data collection was  $42.1 \pm 36.5 \mu g l^{-1}$ . Microscopy analysis of corresponding glutaraldehyde preserved water samples indicated that the phytoplankton communities were dominated by cyanobacteria. Toxic cyanobacteria species, including *Cylindrospermopsis* cf. *raciborskii*, *Raphidiopsis* cf. *curvata*, *Anabaena* cf. *flos-aquae*,

*Anabaena* cf. *circinalis*, and *Microcystis* cf. *aeruginosa*, and non-toxic *Merismopedia* spp. were present in surface water samples for most of the study period. The lake was dominated by alternating blooms of either *Microcystis* or *Anabaena* spp. at all sites.

### 2.3.2 Trends at Site 1

Highest mean Chl *a* concentration ( $48.4 \pm 42.3 \mu\text{g l}^{-1}$ ) occurred at site 1 (S1). However, S1 when compared to other sampling sites, had similar mean Chl *a* concentrations, and cyanobacterial species composition and abundances. Therefore, we focused on S1 as an example of trends occurring at all sampling sites. The relative abundances (%) of the major cyanobacterial genera occurring at S1 were based on calculations from the imaging microscope. Average surface area ratios for individual colonies of *Microcystis* and *Merismopedia* spp. and individual chains of *Anabaena* and *Raphidiopsis/Cylindrospermopsis* spp. were found to be approximately 1.4: 0.7: 10: 1.7, respectively. Figure 2.2 shows the relative abundances (%) of these genera, normalized, to account for surface area differences not resulting from actual differences in abundance (e.g., cell sizes), using these ratios. *Microcystis* sp. dominated over all other phytoplankton, with the exception of December when *Raphidiopsis/Cylindrospermopsis* spp. dominated, and the different genera bloomed at distinct periods (Fig. 2.2).

Winter (between December 2006 and February 2007) was characterized by an absence of *Anabaena* spp. and moderate abundances of *Microcystis* sp. ranging from approximately  $10^2$  to  $10^3$  colonies  $\text{ml}^{-1}$  (Fig. 2.3 C and D). During this period, water temperatures dropped to a minimum of  $9.68^\circ\text{C}$  (Fig. 2.3A), and salinity dropped from the highest salinity (0.66 psu) to the lowest salinity (0.08 psu) (Fig. 2.3A). Toxin analysis for cellular microcystins was not performed on surface water subsamples during this period.

Spring (between March and May 2007), when water temperature increased from  $9.7$  to  $21.5^\circ\text{C}$  (Fig. 2.3A), was distinguished by a sharp increase in *Anabaena* spp. abundance to

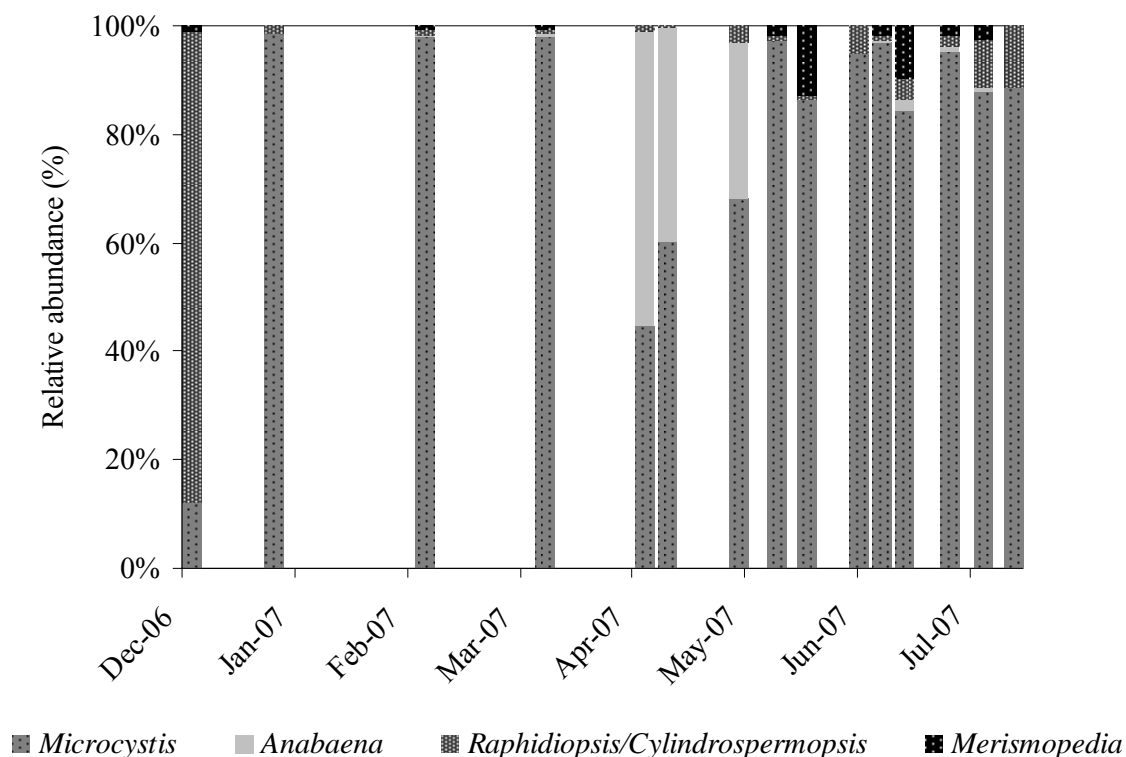


Figure 2.2. Relative abundance (%) of major cyanobacteria genera from samples taken at sampling site 1 (S1) in Lac des Allemands.

approximately  $5.0 \times 10^3$  cells  $\text{ml}^{-1}$  (Fig. 2.3D) on 14 May 2007, representing the largest *Anabaena* spp. bloom during the study. At the same time, *Microcystis* sp. colony abundances declined to minimum values, approximately  $2 \times 10^2$  colonies  $\text{ml}^{-1}$  (Fig. 2.3C). Cellular microcystins reached a high concentration of  $1.26 \mu\text{g MC l}^{-1}$  on 14 May 2007, at the time of the highest occurrence of *Anabaena* spp. (Fig. 2.3 B and D).

Following the decline of the *Anabaena* spp. bloom, the beginning of summer (June 2007) was characterized by a moderate increase in *Microcystis* sp. colonies. As the *Microcystis* sp. concentrations declined, a small increase in *Anabaena* spp. cell abundance occurred. The subsequent decline in concentrations of *Anabaena* spp. cells was accompanied by the largest bloom of *Microcystis* sp. ( $2.4 \times 10^3$  colonies  $\text{ml}^{-1}$ ) on 17 July 2007 (Fig. 2.3C). Within nine days

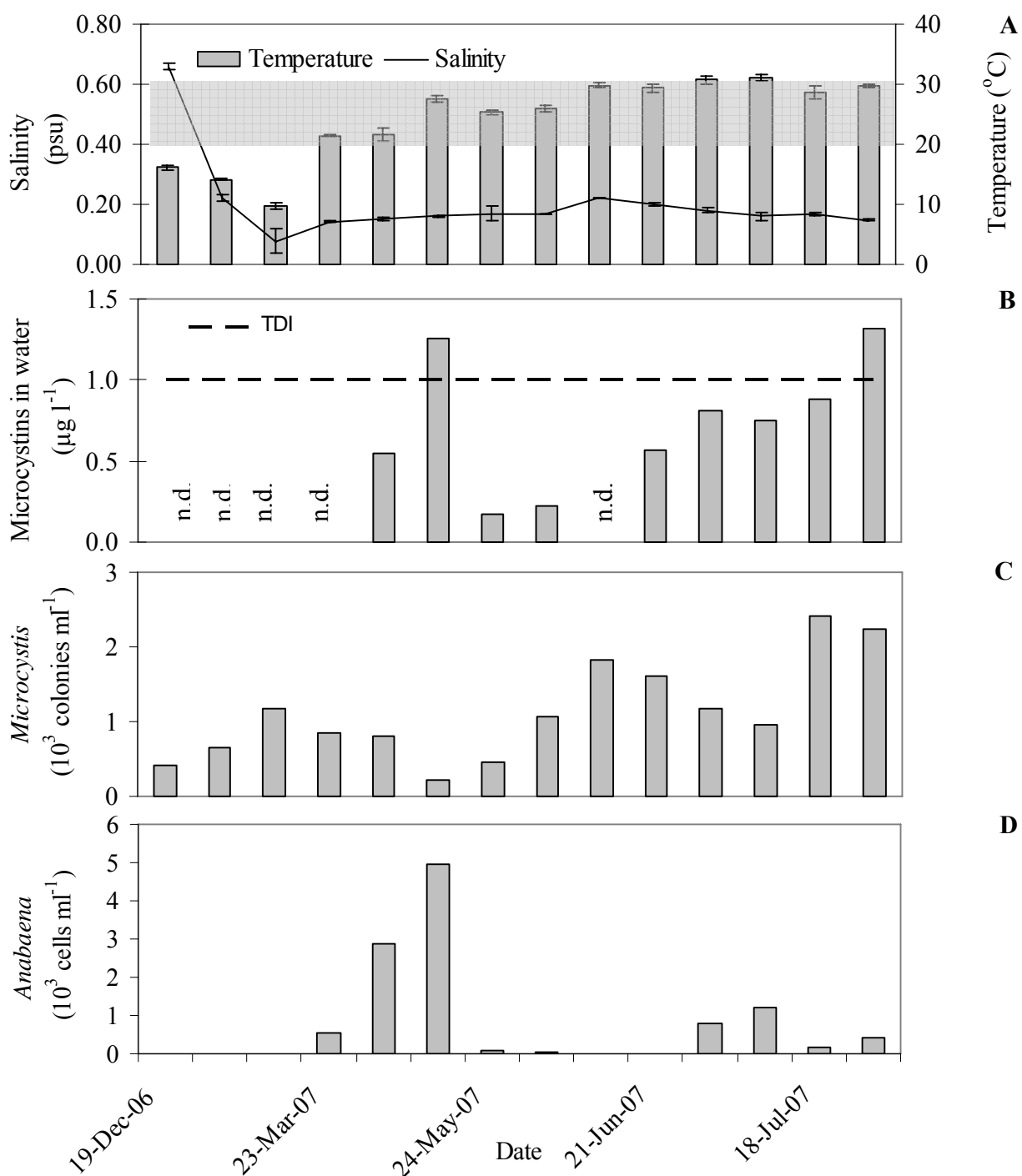


Figure 2.3. Sample site 1 (S1), surface water. (a) Salinity (practical salinity units) (black connected squares) and temperature ( $^{\circ}\text{C}$ ) (gray vertical bars). The gray hollow horizontal bar represents optimal temperature range ( $20\text{--}30^{\circ}\text{C}$ ) promoting cyanobacterial blooms. (b) Concentrations of cellular microcystins in water ( $\mu\text{g MC l}^{-1}$ ) and n.d. is no data. Dashed horizontal line represents World Health Organization (WHO) advisory limit for Tolerable Daily Intake (TDI) of  $1.0 \mu\text{g MC l}^{-1}$ . (c) Abundances of *Microcystis* sp. ( $\text{colonies ml}^{-1}$ ) and (d) *Anabaena* spp. ( $\text{cells ml}^{-1}$ ).

following this *Microcystis* sp. bloom, the highest cellular microcystin concentration ( $1.32 \mu\text{g MC l}^{-1}$ ) occurred on 26 July 2007 (Fig. 2.3B).

### 2.3.3 Microcystins in Surface Water and Crab Tissue

Concentrations of microcystins from surface water subsamples collected at all sites between April and August 2007 ranged from  $0.17$  to  $1.42 \mu\text{g MC l}^{-1}$  (Table 2.4 and Fig. 2.4). Microcystins measured in different blue crab tissue samples, collected between June and July 2007, ranged from not detectable (ND) to  $820 \mu\text{g MC kg}^{-1}$  tissue (wet weight) (Fig. 2.5). Highest microcystin concentrations were found to be  $820 \mu\text{g MC kg}^{-1}$  tissue (wet weight) in hepatopancreas occurring on June 29, 2007,  $65 \mu\text{g MC kg}^{-1}$  tissue (wet weight) in viscera occurring on July 11, 2007, and  $105 \mu\text{g MC kg}^{-1}$  tissue (wet weight) in muscle of a blue crab occurring on June 29, 2007 (Fig. 2.5). Of the blue crab tissues, 77.8% of hepatopancreas, 94.1% of viscera, and 37.5% of muscle had detectable concentrations of microcystins. Microcystin concentrations found in surface water samples and in all blue crab tissues are summarized in Table 2.4.

Table 2.4. Microcystin concentrations for water ( $\mu\text{g MC l}^{-1}$ ) and blue crab ( $\mu\text{g MC kg}^{-1}$ ) samples taken from Lac des Allemands, 2007.

<b>Sample Type</b>	<b><i>n</i></b>	<b>Avg + Std Dev</b>	<b>Range</b>	<b>% Detects</b>
Water	19	$0.77 \pm 0.37$	(0.17-1.42)	100%
Crab Tissue <sup>1</sup> :				
Hepatopancreas	18	$79.7 \pm 189$	(ND-820)	77.8%
Viscera	17	$24.1 \pm 20.5$	(ND-65)	94.1%
Muscle	8	$21.3 \pm 37.5$	(ND-105)	37.5%

1: Crab size range 100-196 mm, caught during recreational and commercial harvest season between June and July 2007.

*n* represents number of samples analyzed.

ND, not detectable.



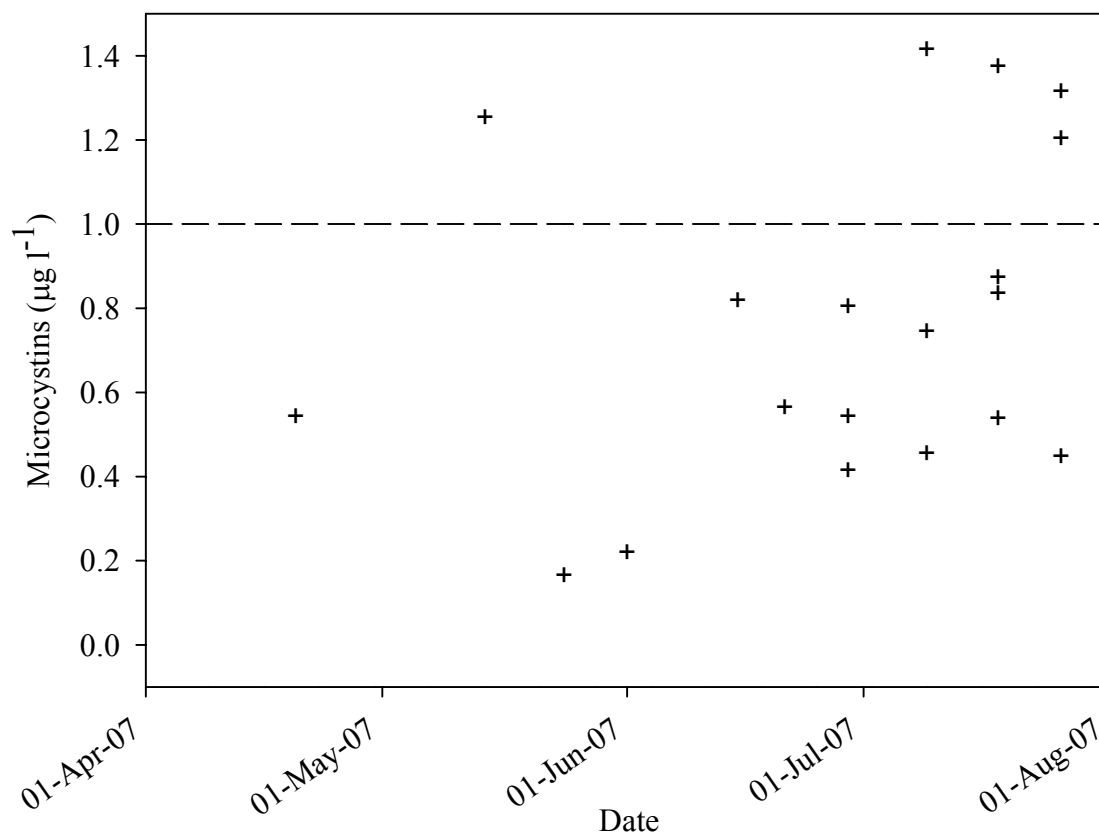


Figure 2.4. Concentration of microcystins ( $\mu\text{g MC l}^{-1}$ ) ( $n=19$ ) in surface water of sampling sites 1, 2, 3, and 4. Dashed horizontal line represents World Health Organization (WHO) advisory limit for Tolerable Daily Intake (TDI) of  $1.0 \mu\text{g MC l}^{-1}$ .

### 2.3.4 Bivalve Prey in Crab Gut Samples

Bivalve shell remnants were visible in 50% of gut samples analyzed. Plant material, sediment, crustacean remnants, and unidentified organic material were also visible.

## 2.4 Discussion

Lac des Allemands is the freshwater end member of the Barataria estuary and is hyper-eutrophic (Carlson, 1977). High Chl *a* concentrations suggest that high nutrient loads may be reaching and fertilizing the upper Barataria estuary. Cyanobacteria are particularly successful in such eutrophic estuaries due to their tolerance of large changes in water chemistry, including pH and salinity, coupled with an ability of some to fix nitrogen in specialized heterocysts (Dokulil

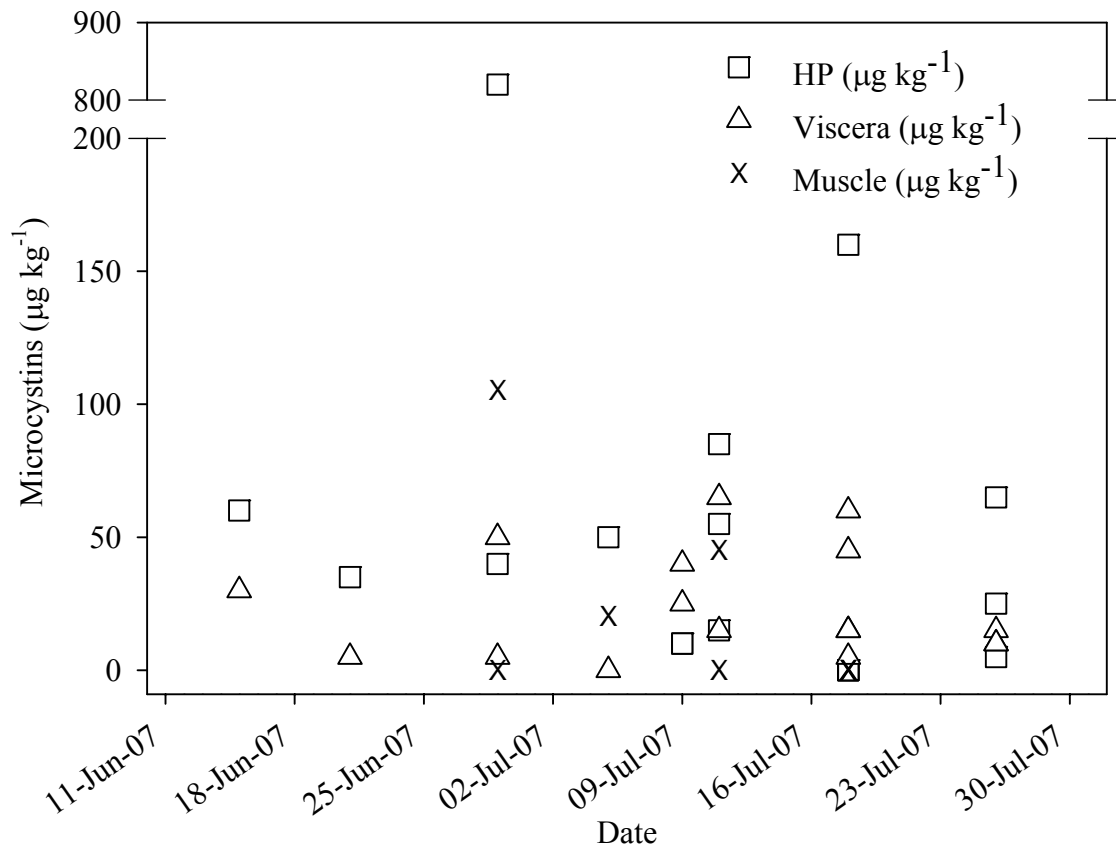


Figure 2.5. Concentration of microcystins ( $\mu\text{g MC kg}^{-1}$ ) in hepatopancreas, viscera, and muscle tissues of *Callinectes sapidus* collected at sampling sites 1, 2, 3, and 4.

and Teubner 2000). Vegetative cells of cyanobacteria can often differentiate into heterocysts or akinetes, thick-walled resting cells, allowing blooms to persist during extreme environmental conditions. Optimal temperatures for photosynthesis in cyanobacteria occur between  $20^{\circ}\text{C}$  and  $30^{\circ}\text{C}$  (Konopka and Brock, 1978; Robarts and Zohary, 1987), which are temperatures higher than those preferred by green algae and diatoms (Msagati et al., 2006). These characteristics of cyanobacteria allow for their dominance in estuarine phytoplankton communities especially during summer at low latitudes. The present findings support these conclusions from previous studies, with cyanobacteria being the most abundant phytoplankton group present during this study and the highest concentrations of *Microcystis* and *Anabaena* spp. occurring after surface water temperatures exceeded  $20^{\circ}\text{C}$ . Inverse relationships between abundances of *Microcystis* and

*Anabaena* spp. indicate that different environmental factors may control phytoplankton community structure.

The detailed observations of trends occurring at S1 indicate that both *Anabaena* and *Microcystis* spp. contribute to cellular microcystin concentrations in surface waters (Fig. 2.3B, C, and D). Toxin production by *Microcystis* and *Anabaena* genera was detected in surface waters from all four sampling sites, with the maximum cellular microcystin concentration of 1.42  $\mu\text{g MC l}^{-1}$  occurring in July 2007. This value exceeded the WHO tolerable daily intake (TDI) limit of 1.0  $\mu\text{g MC l}^{-1}$  (WHO, 1998). This TDI limit was exceeded in 26.3% ( $n=19$ ) of particulate surface water samples from all four sites (Fig. 2.4).

The data confirm accumulation of microcystins in hepatopancreas, viscera, and muscle tissue of the demersal blue crab, *C. sapidus*, during presence of toxic cells in surface waters. Crabs commercially harvested and supplied to a local seafood market (purchased and used for spike and recovery experiments in this study) were also found to be contaminated with low levels of microcystins. Variation in microcystin concentrations found in blue crab tissues likely reflects spatial and temporal changes in abundances of *Microcystis* and *Anabaena* cells in the water column, time lagged accumulation in blue crab (Ibelings and Havens 2007 and references therein), changes in microcystin depuration rates in response to environmental parameters (e.g., temperature (Ibelings and Havens 2007)), and the mobility and opportunistic feeding behavior by the crabs.

The data indicate that the main route of exposure to microcystins is through ingestion of contaminated prey as suggested by high concentrations in hepatopancreas, a vital part of the digestive system, and viscera. In the Barataria estuary system of Louisiana, clams and mussels inhabit the fresh to brackish water regions of the mid and upper estuary, while oysters are restricted to the mid and lower parts of the estuary where salinity is higher. Bivalve shell

remnants were visible in multiple gut samples, implicating these filter-feeding prey as likely vectors of microcystins to blue crabs. The overlapping distribution and abundance of blue crabs and their prey may enhance the short dietary link between harmful algal blooms, blue crabs, and human consumers of blue crabs in the upper regions of this estuary.

Earlier studies have shown that benthic freshwater bivalves accumulate high levels of microcystins (Chen and Xie, 2007) and serve as likely vectors for these toxins to blue crabs. Amorim and Vasconcelos (1999) demonstrated that the depuration of microcystins by the mussel *Mytilus galloprovincialis* was slow, with low levels of microcystins present in feces two weeks after initial exposure. In the same study, mussels also re-ingested microcystin-contaminated feces, increasing the persistence of the toxins after toxic blooms end. Other studies, however, have shown depuration to be rapid, but have also shown that depuration can be incomplete and temperature dependent (Ibelings and Havens 2007). Ozawa et al. (2003) suggested that residual microcystin accumulated in tissues of *Sinotaia histrica*, a freshwater snail, during the autumn and winter would still be detectable before cyanobacterial blooms developed the following spring. These results have implications for blue crabs and other higher trophic level organisms that consume bivalves in areas where cyanobacterial blooms have recently occurred, and indicate that monitoring for microcystins presence in surface waters is not sufficient for determining potential risk to these fisheries. Analysis of multiple deceased catfish, opportunistically collected from the surface of Lac des Allemands during this study, also revealed low levels of microcystins within the stomach and intestines (Garcia et al., unpublished data). In conjunction with our toxin data for blue crab tissues, this is not a surprising finding, as blue crabs and catfish employ similar feeding strategies. However, these deceased catfish could also serve as a potential food source for blue crabs, though toxin transfer through a filter-feeding prey is more

plausible. Continuous monitoring for cyanobacterial toxins in water, sentinel organisms, and tissues of organisms harvested for human consumption should be considered.

Laboratory studies by Dewes et al. (2006) on the estuarine intertidal burrowing crab, *Chasmagnathus granulatus*, demonstrated that even relatively low internal concentrations ( $13.2 \pm 0.56 \mu\text{g MC kg}^{-1}$ ) of microcystins can induce physiological and biochemical disturbances in the form of oxidative stress and the lowering of glycogen content within the hepatopancreas. Mean concentration of microcystins in all blue crab hepatopancreas analyzed during our study was  $79.7 \pm 189 \mu\text{g MC kg}^{-1}$  (wet weight) (Table 2.4). This mean is six fold the concentration shown to cause detrimental impacts on *C. granulatus* when administered aqueous extracts of *Microcystis aeruginosa*. However, the high variation in the mean hepatopancreas toxicity is coming from a single data point,  $820 \mu\text{g MC kg}^{-1}$  (wet weight), which occurred on June 29, 2007 at S1 (Fig. 2.5), and in excluding this point we get a mean of  $36.2 \pm 41.7 \mu\text{g MC kg}^{-1}$  (wet weight), which may represent a more commonly encountered level of toxicity in the hepatopancreas. Although this mean concentration initially appears less dangerous, the potential impacts of continuous or repeated exposure at these levels on aquatic organisms, remains poorly understood. The present results indicate that continued eutrophication and toxic algal blooms in the Barataria estuary may have adverse impacts on blue crab health, ultimately impacting blue crab fisheries and human health.

Figure 2.6 depicts the calculated EDI values for a 60 kg human consuming hepatopancreas, muscle, or all edible tissues (including viscera) from 6 crabs (300 g of tissue, as previously assumed), which may represent an underestimate of EDI values for some samples due to the previous assumption that no toxin was present in tissue samples that were not directly measured during this study. The maximum value for microcystins in tissue samples ( $820 \mu\text{g MC kg}^{-1}$  wet weight) occurred from the hepatopancreas of a blue crab captured on June 29, 2007 at

S1 (Fig. 2.5), which represents an estimated daily intake (EDI) concentration of  $0.36 \mu\text{g MC kg}^{-1}$  of body weight  $\text{day}^{-1}$  (Fig. 2.6). This EDI is nine times greater than the WHO guideline TDI of  $0.04 \mu\text{g MC kg}^{-1}$  body weight  $\text{day}^{-1}$  (WHO, 1998). Also known as the "mustard," the hepatopancreas is consumed by many people either as soft shelled crab, where muscle tissue and viscera parts are also consumed, or otherwise, in gumbos or as a delicacy. However, the hepatopancreas, which functions as the liver and pancreas, often contains toxic contaminants (Sastre et al., 1999) and is generally not recommended for consumption. As expected, hepatopancreas samples generally contained the highest microcystin concentrations of the tissue types analyzed during this study. However, because blue crab muscle tissue is generally consumed more often and in higher quantity, EDI calculation for this tissue becomes more important than of hepatopancreas, despite lower levels of toxicity. The maximum value for microcystins in muscle tissue, from the same blue crab caught on June 29, 2007 at S1, was  $105 \mu\text{g MC kg}^{-1}$  (wet weight) (Fig. 2.5), representing an EDI of  $0.46 \mu\text{g MC kg}^{-1}$  body weight  $\text{day}^{-1}$  (Fig. 2.6), a value more than ten times the WHO-TDI guideline. Using the average microcystin values found for each crab tissue type (Table 2.4) and equation 1, we can consider the EDI concentrations as a function of human body weight and the number of whole crabs consumed per day (Fig. 2.7). In using average microcystin values for hepatopancreas, viscera, and muscle, we assume that all crabs consumed have the same ratios of toxin available, which we have shown in this study not to be true. However, using these averages we can provide a conservative estimate of public health risk. As an example, a 70 kg individual can consume the edible tissues (hepatopancreas, viscera, and muscle) of up to 2 crabs per day before exceeding the WHO guideline TDI of  $0.04 \mu\text{g MC kg}^{-1}$  body weight  $\text{day}^{-1}$  (Fig. 2.7). Toxin extraction efficiencies for hepatopancreas and viscera were moderate to high, however, recovery rates for muscle tissues

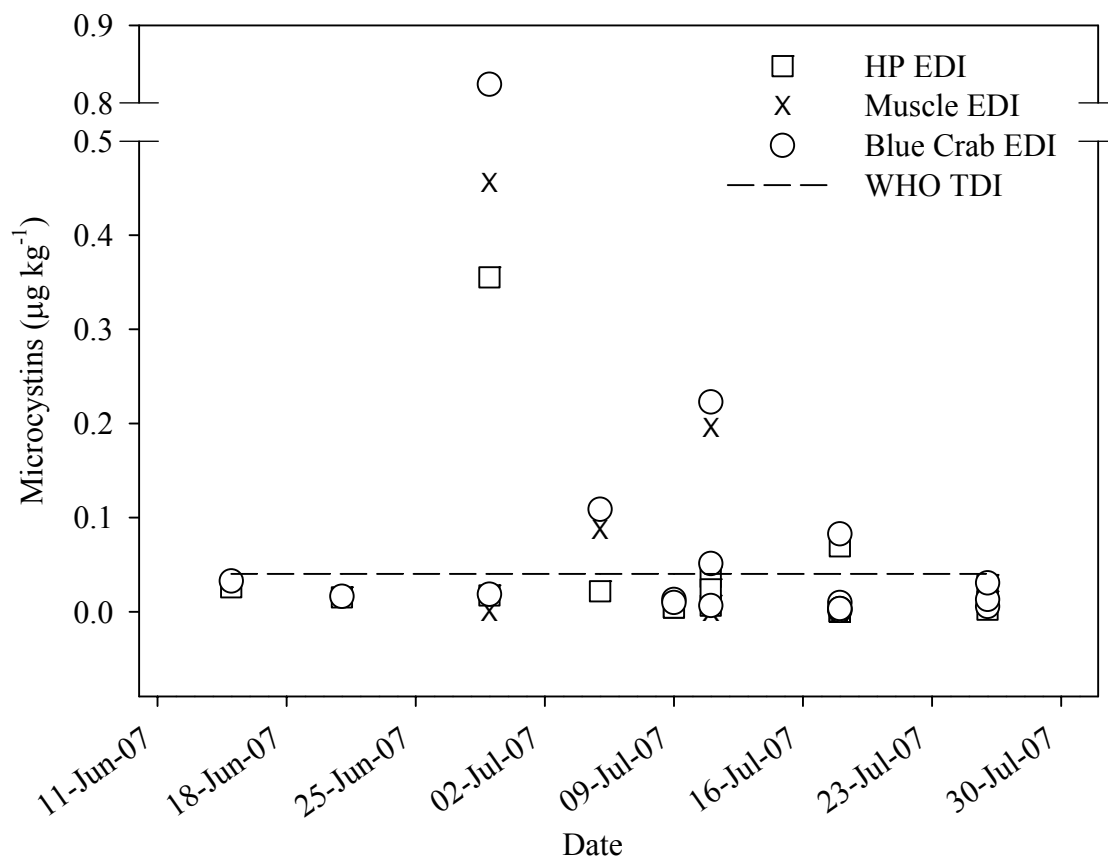


Figure 2.6. Estimated Daily Intake (EDI) concentrations ( $\mu\text{g MC kg}^{-1}$ ) for a 60 kg adult consuming 300 g of entire crab (all edible tissues) (Blue Crab EDI), hepatopancreas, or muscle tissues per day. Dashed line represents World Health Organization (WHO) Tolerable Daily Intake (TDI) value of  $0.04 \mu\text{g MC kg}^{-1}$  body weight  $\text{day}^{-1}$ .

were much lower. If these rates were corrected for, EDI estimates would double for muscle tissues where toxin concentrations were detected. However, we chose not to correct data for recovery rates for technical reasons discussed later, and thereby hope to present more conservative estimates of potential risk.

It should be noted that only 11.1%, 0%, and 37.5% of hepatopancreas, viscera, and muscle tissues, respectively, had such high microcystin concentrations that calculated intake exceeded the advisory TDI of  $0.04 \mu\text{g MC kg}^{-1}$  body weight  $\text{day}^{-1}$  (WHO, 1998). However, it is also important to note that chronic exposure at low concentrations of microcystins may increase health effects. Ito et al. (1997) showed that repeated exposure at sub-lethal concentrations

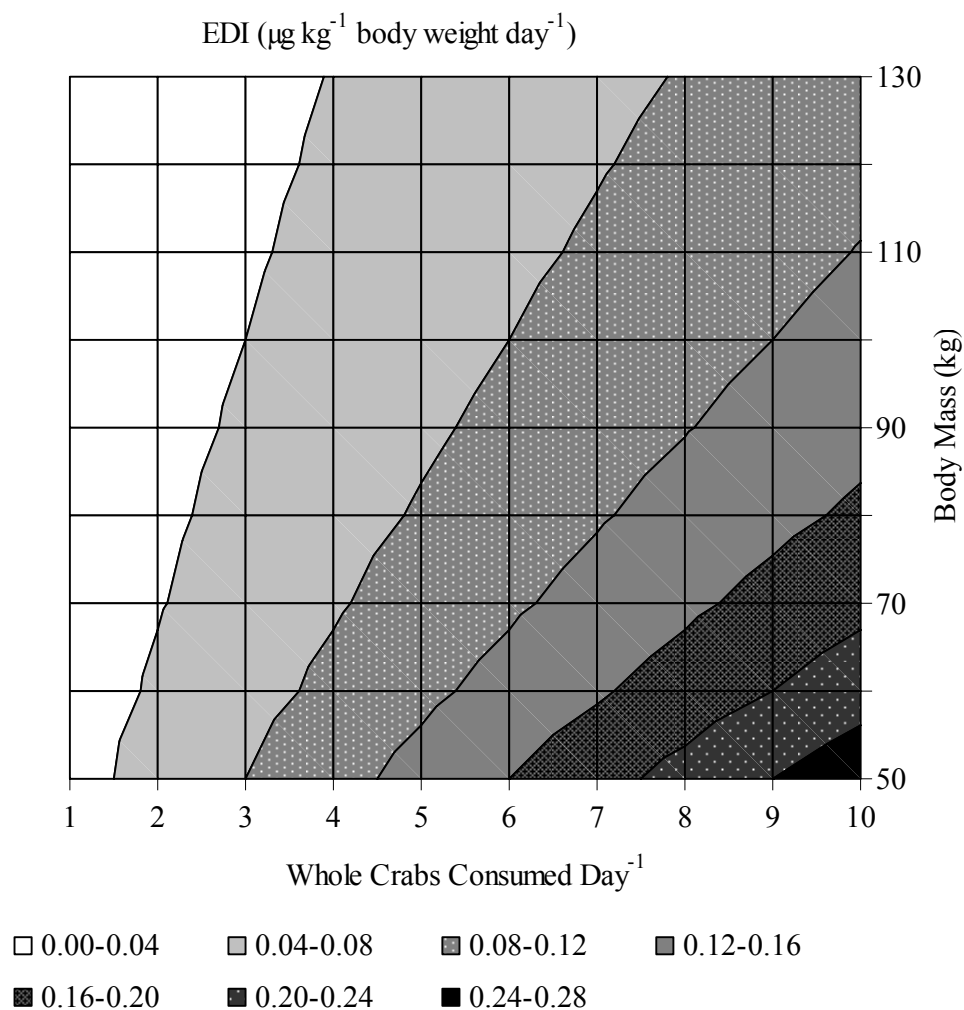


Figure 2.7. Estimated Daily Intake (EDI) concentrations ( $\mu\text{g MC kg}^{-1}$ ) as a function of body mass (kg) and number of whole crabs consumed (edible tissue from hepatopancreas, viscera, and muscle) per day. World Health Organization (WHO) Tolerable Daily Intake (TDI) value of  $0.04 \mu\text{g MC kg}^{-1} \text{ body weight day}^{-1}$  is reached or exceeded for areas shaded in gray or black.

promoted the formation of neoplastic nodules (tumors) in mice liver. Also, suggested TDI values are not applicable to the elderly and children, who may be more susceptible to the toxins at lower levels. Since our blue crab harvest occurred during a period of relatively low average Chl *a* concentrations ( $23.5 \pm 5.0 \mu\text{g l}^{-1}$ ) in surface water, toxin data for blue crab tissue may not be representative of periods of high chlorophyll concentrations when toxicity could potentially increase, depending on the toxic cyanobacteria species present, the growth phase of toxic cells,



environmental parameters (Song et al., 1998), and the corresponding efficiency of microcystin transfer to blue crabs through the food web. Although, all phytoplankton contain chlorophyll *a*, it is generally considered a poor indicator for cyanobacterial blooms. Use of several carotenoids and the phycobilliproteins is preferred for the evaluation of cyanobacteria presence and abundance. Therefore, further studies are needed to better characterize the temporal variation of toxic cyanobacteria in surface waters and corresponding microcystin concentrations in blue crab tissues.

For comparison to established WHO guidelines, the concentrations found in this study good cross-reactivity with most cyanobacterial cyclic peptide congeners (Fischer et al., 2001), but does not allow for the further description of congeners and detoxication metabolites present. Microcystin-LR is considered the most common of the variants of microcystins (Fischer et al., 2001), and it is among the more toxic variants (Carmichael, 1997). Also, microcystins were determined following standard methanol extraction; a method that will not account for microcystin covalently bound to protein phosphatase enzymes, making the complexes unavailable for toxin detection. Williams et al. (1997) found that ~60% of the total radio-labeled microcystin-LR in salmon liver was not extractable with methanol five hours following intraperitoneal injection, likely due to covalent bonding to protein phosphatases. This extraction method may explain the lower recovery rates in tissue samples, thereby significantly underestimating those microcystin concentrations. However, complexes of microcystin covalently bound to protein phosphatase enzymes are thought to be less toxic when compared to unbound microcystins, but questions remain largely unanswered (Ibelings and Havens, 2007). Therefore, microcystin concentrations found in tissues and EDI calculations must be interpreted with care.

For the purposes of analyzing risk associated with blue crab consumption, we relate the results of this study to the provisional guidelines suggested by the WHO for both drinking water and edible tissue. Unlike studies on marine algal toxins where hazards associated with the ingestion of contaminated water or organisms (e.g., shellfish) are well understood, relatively little work has been done on freshwater algal toxins. Therefore, limits suggested by the WHO for water and tissue samples are provisional. However, by comparing our findings to WHO-TDI limits, we can evaluate the potential risk associated with consumption of microcystin-contaminated blue crab taken from a hyper-eutrophic estuary, and thereby characterize blue crab as a potential vector for cyanobacterial toxins to humans.

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## CHAPTER 3

### VARIABLE ACCUMULATION AND OXIDATIVE STRESS WITHIN BLUE CRAB (*CALLINECTES SAPIDUS*) ORALLY EXPOSED TO MICROCYSTIN-LR AND -RR

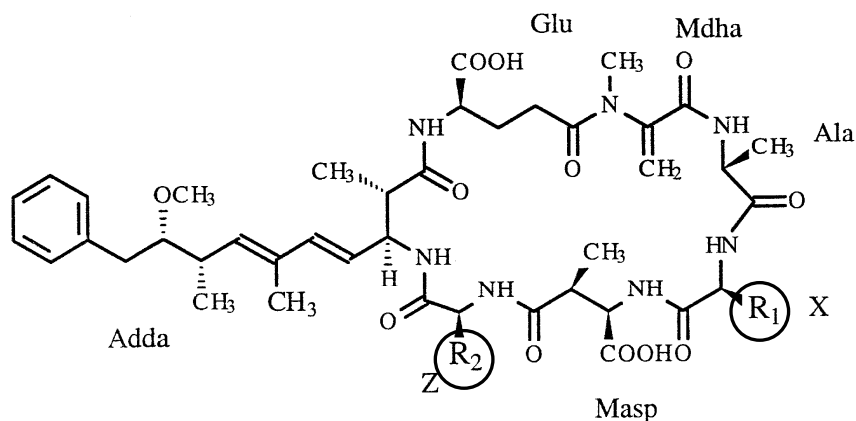
#### 3.1 Introduction

Intense cyanobacterial blooms are increasingly occurring in eutrophic freshwater systems worldwide (Codd, 2000). Some cyanobacteria are of particular concern due to their production of potent hepatotoxins and tumor promoters (Ito et al., 1997), known as microcystins. Exposure to water contaminated with these compounds has caused multiple deaths of domestic and wild animals (Carmichael, 1994), and consumption of water or aquatic animals from microcystin contaminated systems may pose a threat to human health (Chen et al., 2009; Garcia et al., 2010).

Predation on non-selective filter-feeding bivalves, such as *Rangia cuneata* (Darnell, 1958), make blue crabs (*Callinectes sapidus*) vulnerable to phycotoxin exposure. Previous studies by Garcia et al. (2010), Galván et al. (unpublished data), and Morrison et al. (unpublished data) have found microcystin concentrations in hepatopancreas, viscera, and muscle of blue crab taken from a eutrophic freshwater lake in southern Louisiana. Louisiana is the largest producer of blue crabs in the United States (McKenzie et al., 1995; Guillory and Perret, 1998), with more than one-quarter of the nation's blue crab production occurring in Louisiana in 2001 (Lester et al., 2005). Thus, understanding the dynamics of microcystin accumulation, depuration, and their potential impacts on blue crabs is of particular importance, especially as they may serve as potential vectors of these potent toxins.

Microcystins are monocyclic heptapeptides containing D-alanine, D-glutamic acid,  $\beta$ -linked D-ethyry-  $\beta$ -methyiaspartic acid (MeAsp), N-methyl dehydroalanine (MDha), (2S,3S,8S,9S)-3-amino-9-methoxy-2-6,8-trymethyl, 10-phenyldeca-4,6-dienoic acid (Adda), and two variable L- amino acids present at positions 2 and 4 that differentiate between the

microcystin congeners. Of the greater than 60 congeners of microcystin that have been described, most studies focus on microcystin-LR, which has Leucine and Arginine at the 2 and 4 positions (Fig. 3.1). Microcystin-LR is among the most toxic and commonly found of the microcystin congeners, with a  $LD_{50}$  in mice of  $50 \mu\text{g kg}^{-1}$  (Spoof 2005). However, multiple studies indicate that the less toxic microcystin-RR congener, containing Arginine at both positions (Fig. 3.1), resists degradation during digestion with greater success (Ozawa et al., 2003; Xie et al., 2004; Jang et al., 2004; Chen and Xie 2005). Despite having a lower toxicity ( $LD_{50}$  in mice of  $600 \mu\text{g kg}^{-1}$ ) relative to microcystin-LR (Spoof 2005), physiological impacts from microcystin-RR may be more severe depending on the mechanism of exposure. Garcia et al. (2010) found that the main route of exposure to microcystins in the blue crab, *Callinectes sapidus*, was likely through the ingestion of contaminated prey. This indicates that microcystin-RR may have more severe impacts on blue crab fisheries and human consumers of blue crab muscle tissues, as it may be assimilated into tissues beyond those of the digestive system.



Microcystin-LR: R1 = Leucine; R2 = Arginine

Microcystin-RR: R1 = R2 = Arginine

Figure 3.1. Structure of microcystins (modified from Trogen et al., 1998) showing side chains of variable L- amino acids present at positions 2 and 4 that differentiate microcystin-LR and -RR congeners.



Microcystin-LR and -RR toxicity occurs through covalent binding to serine/threonine protein phosphatases 1 and 2A, inhibiting phosphate group removal. The resulting hyper-phosphorylated cellular state causes hepatic hemorrhaging and failure (Falconer et al., 1981; MacKintosh et al., 1990). However, oxidative damage has been implicated as an alternative mechanism of microcystin toxicity, made evident through several biomarkers including increases in antioxidant activity, reactive oxygen species (ROS, highly reactive molecules containing oxygen ions), and lipid peroxide (LPO) levels. Microcystins conjugate to tripeptide glutathione (GSH), an important part of the antioxidant defense system, thereby decreasing the intracellular GSH supply and its protective capacity. Glutathione *S*-transferase (GST) catalyzes the conjugation of microcystins and ROS damaged cellular components with GSH for detoxification (Storey 1996; Pflugmacher et al., 1998), with increased GST activity indicating increased demand for GSH. Oxidative stress may occur in tissues when the activity of these antioxidant defense systems decrease or are overwhelmed by ROS generation (Packer 1995). Lipid peroxidation is the degradation of lipids caused by ROS, producing highly unstable hydroperoxides of saturated and unsaturated lipids, causing cell damage (Amado and Monserrat, 2010).

Many studies have measured oxidative stress biomarkers in vertebrate and invertebrate aquatic organisms in response to different exposure lengths, routes, and doses of the various microcystin congeners. Bláha et al. (2004) observed elevated GSH levels in silver carp exposed for 25 days to living populations of microcystin-producing cyanobacteria, which indicated increased antioxidant demand due to oxidative injury. In tilapia fish intraperitoneally exposed to a single dose of 500  $\mu\text{g kg}^{-1}$  of either microcystin-LR or -RR, significant increases were seen from both toxins in antioxidant enzymatic activities of fish sacrificed after 7 days (Prieto et al., 2006). However, in the same study, levels of lipid peroxidation changed only in response to

microcystin-LR. In invertebrates, increases in Glutathione *S*-transferase (GST) activity have been documented in gills and hepatopancreas of the estuarine crab, *Chasmagnathus granulatus*, following exposure to aqueous extracts of microcystins (Vinagre et al., 2003; Pinho et al., 2003; Dewes et al., 2006). Pinho et al. (2005) found significantly increased lipid peroxide levels in the hepatopancreas of the same crab species following 7 days of daily oral doses ( $5.32 \mu\text{g kg}^{-1} \text{ day}^{-1}$ ) of *Microcystis aeruginosa* extracts. However, significant daily temporal variations can be seen in antioxidant defenses and lipid peroxidation levels in the gills and the hepatopancreas of *Chasmagnathus granulata* (Maciel et al., 2004), indicating that further confirmation of these oxidative stress biomarkers may be needed.

Multiple studies have considered that ecdysis (molting) may be a mechanism for decreasing body burdens of heavy metals in arthropods and insects (Keteles and Fleeger, 2001; Raessler et al., 2005; Bergey and Weis, 2007), although results from these studies are often contradictory. Nevertheless, the depuration of other toxicants during the ecdysis process has remained relatively unexplored and processes occurring within the hypodermis may serve as a potential detoxification strategy in crustaceans. The hypodermis is the layer of skin cells located directly underneath the exoskeleton (carapace) of crustaceans. Prior to ecdysis, the hypodermis separates from the old exoskeleton and a new cuticle is deposited. The hypodermis becomes an area of exchange, as much of the old cuticle is reabsorbed and transported through the hypodermis to the hemolymph. During this process, blue crabs also develop new gill membranes and shed previous ones. Ingestion of whole *Microcystis aeruginosa* cells and exposure to microviridin J, a toxic compound associated with *Microcystis* spp. cells, was found to inhibit molting processes in *Daphnia* spp. likely by diminishing protease activity (Rohrlack et al., 2001, 2004). Thus, microcystins and the various cyanobacterial cell-associated compounds may have significant impacts on these crustacean growth processes.

As toxic cyanobacterial blooms are predicted to increase in response to accelerated eutrophication, understanding tissue burdens of microcystins and their impacts on oxidative stress biomarkers is becoming increasingly important to further assess the implications these natural toxicants may have on commercially important fisheries. The present research was conducted on blue crab (*Callinectes sapidus*) taken from multiple freshwater lakes in southern Louisiana. The objectives of this study were to evaluate the accumulation and distribution of microcystin-LR and -RR in various organs (hepatopancreas, viscera, muscle, hypodermis, and gills) of blue crabs orally exposed under laboratory conditions. Additionally, hepatopancreas of blue crab were used for analysis of oxidative stress using lipid peroxidation biomarkers.

### **3.2 Materials and Methods**

#### **3.2.1 Chemicals**

Microcystin-LR and -RR (purity  $\geq 95\%$ ) were purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). Standard stock solutions and working standard solutions were prepared in crustacean saline solution (10 mM  $\text{MgCl}_2$ ; 335 mM  $\text{NaCl}$ ; 16.6 mM  $\text{CaCl}_2$ ; 5 mM  $\text{H}_3\text{BO}_3$ ; 10 mM  $\text{KHCO}_3$ ; 8 mM  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ ; pH adjusted to 7.6; Vinagre et al., 2003).

#### **3.2.2 Blue Crab Acquisition and Housing**

Blue crab, *Callinectes sapidus*, were acquired from commercial fishermen from the freshwater lakes ( $\leq 1-3$  psu), Lac des Allemands and Lake Pontchartrain, located in southeastern Louisiana, USA, between October and December 2009. Sediment and water column samples from Lac des Allemands taken during this period were found to have relatively low cyanobacterial biomass (Galván et al., unpublished data) and further visual observations of water color indicated that no intense cyanobacterial blooms were present at either site during crab collection. The blue crabs were collected using wire crab traps baited with locally caught fishes. Forty-five adult male crabs of similar carapace size and weight ( $16.89 \pm 1.30$  cm and  $276.54 \pm$

46.60 g, respectively) were selected. Additional selection criteria for crabs included use of both claws, lack of external parasites, and no visible injuries to carapace. Crabs meeting these criteria were transported in aerated coolers containing water soaked burlap sacs. Blue crabs were transferred to 10 gallon aquariums with DI water maintained at a controlled salinity and temperature (1 psu and 25°C, respectively). Aquariums were continuously aerated and filtered using undergravel filters covered with a 2.54 cm layer of aquarium gravel. Blue crabs were acclimated to experimental conditions for at least one week, and during this period, were fed once daily with whole, thawed silverside fish, commercially available as frozen fish food (Ocean Nutrition). Blue crabs were not fed 24 hours prior to the start of each experiment in order to ensure that crabs would consume the entire fish presented during the experimental period.

### **3.2.3 Preliminary Mortality and Behavioral Abnormality Observations**

In order to establish if death or behavioral changes were likely to occur as a result of exposure to either microcystin-LR or -RR, a preliminary experiment was performed. After acclimation, 2 healthy crabs were randomly chosen. Carapace width (including lateral spines) was measured and crabs were weighed prior to exposure and before being sacrificed. In order to increase likelihood of observing behavioral changes from microcystin exposure, crabs were exposed intramuscularly (i.m.) as this is a more direct and lethal route of exposure. One crab was given a dose of 1000 µg of microcystin-LR kg<sup>-1</sup> crab body weight<sup>-1</sup> i.m., while the other crab was given 2000 µg of microcystin-RR kg<sup>-1</sup> crab body weight<sup>-1</sup> in the same manner. A higher dose was chosen for microcystin-RR because of its lower toxicity compared to microcystin-LR (Ibelings and Havens, 2007 and references therein). Crabs were observed for behavioral changes for 24 hours or until either crab died.

### 3.2.4 Microcystin-LR and -RR 48-Hour Single-Exposure Experiment

In order to evaluate the acute effects of natural route exposure to various doses of microcystin-LR and -RR and characterize tissue accumulation within blue crabs, 48-hour single-exposure experiments were performed. After acclimation, 36 healthy blue crabs were randomly divided into 4 groups, 2 control groups and 2 microcystin treatment groups. Carapace width (including lateral spines) was measured and crabs were weighed at the beginning of each experiment and prior to crabs being sacrificed. Control groups were fed a single whole, thawed silverside fish injected with crustacean saline solution, with one control group sacrificed immediately following feeding (zero hours ( $t = 0$ )) and the other group sacrificed after 48 hours (forty-eight hours ( $t = 48$ )). Crabs in the first treatment group were exposed orally to microcystin-LR by feeding each a single whole, thawed silverside fish injected with a dose of 10, 100, 500, or 1000  $\mu\text{g kg}^{-1}$  of crab body weight<sup>-1</sup>, and were sacrificed 48 hours after consuming the fish. Crabs in the second treatment group were orally exposed to microcystin-RR by feeding each a single whole, thawed silverside fish injected with a dose of 10, 100, 500, or 1000  $\mu\text{g kg}^{-1}$  of crab body weight<sup>-1</sup>, and were sacrificed 48 hours after consuming the fish. Control and treatment crabs were observed during feeding to ensure consumption of entire fish without excessive handling by the crab. Crabs that did not consume fish immediately or completely were eliminated from the experiment. Each control and treatment was replicated three times. Hepatopancreas, muscle, viscera, and hypodermis were dissected immediately from freshly sacrificed crabs and either frozen ( $-20^{\circ}\text{C}$ ) for toxin analyses, extracted and frozen ( $-80^{\circ}\text{C}$ ) for biochemical measurement. Visible pieces of crab feces were collected from each aquarium and frozen ( $-20^{\circ}\text{C}$ ) for toxin analyses. After stirring aquarium water and gravel, one liter of aquarium water was filtered through a glass fiber filter (Whatman GF/F) and filters (particulate toxin) and dissolved filtrate (dissolved toxin) were frozen ( $-20^{\circ}\text{C}$ ) for toxin analyses.

### 3.2.5 Microcystin-RR Repeated-Exposure Experiment

In order to evaluate the effects of repeated doses of microcystin-RR at a concentration likely to be encountered in common blue crab prey (mean *Rangia cuneata* microcystin concentrations were  $\sim 100 \mu\text{g kg}^{-1}$  (Galván et al., unpublished data)), 7-day (168-hour ( $t=168$ )) daily-exposure experiments were performed. The microcystin-RR congener was chosen as it is thought to accumulate in tissues with greater success relative to microcystin-LR, and because few studies have evaluated the effect of this congener on invertebrates. Following acclimation, healthy blue crabs were randomly divided into 2 groups, 1 control group ( $n=3$ ) and 1 treatment group ( $n=4$ ). Carapace width (including lateral spines) was measured and crabs were weighed at the beginning of each experiment and prior to crabs being sacrificed. Crabs in the control group were each fed a single whole, thawed silverside fish, injected with crustacean saline solution, daily for 7 days. Crabs in the treatment group were each fed a single whole, thawed silverside fish, injected with a dose of  $50 \mu\text{g microcystin-RR kg}^{-1}$  crab body weight<sup>-1</sup>, daily for 7 days. Hepatopancreas, muscle, viscera, hypodermis, and gills were dissected immediately from freshly sacrificed crabs and either frozen ( $-20^{\circ}\text{C}$ ) for toxin analyses, extracted and frozen ( $-80^{\circ}\text{C}$ ) for biochemical measurement. Visible pieces of crab feces were collected from each aquarium and frozen ( $-20^{\circ}\text{C}$ ) for toxin analyses. After stirring aquarium water and gravel, one liter of aquarium water was filtered through a glass fiber filter (Whatman GF/F) and filters and dissolved filtrate were frozen ( $-20^{\circ}\text{C}$ ) for toxin analyses.

### 3.2.6 Microcystins Analyses

#### 3.2.6.1 Microcystins Extraction from Blue Crab Tissues

Microcystin extraction was performed on hepatopancreas, muscle, viscera, hypodermis, and gills of blue crab, according to Garcia et al. (2010). Tissue samples were homogenized separately for each tissue type using a hand-held tissue homogenizer. A 3 g aliquot of

homogenized tissue was then collected and extracted using 1:4 ratio of tissue to 75% methanol, briefly vortexed, sonicated (2 min, 30–40 W), and centrifuged (20 min, 3500 rpm). Supernatant was collected, filtered through 0.2 mm syringe filter (Corning), and stored at -20 °C until analyses by immunoassay.

### **3.2.6.2 Microcystins Extraction from Filtered Particulate, Feces, and Dissolved Samples**

Microcystins extractions of particulate water samples and feces were performed at room temperature according to Garcia et al. (2010). Methanol: water: acetic acid (50:49:1, v/v, Boyer, 2008) extraction solution (5 ml) was added to filters and feces, which were vortexed (1 min), sonicated (2 min, 30–40 W) in an ice bath using a sonicator probe, and centrifuged (10 min, 3000 rpm) to remove debris. The supernatant was removed and filtered through a 0.2 mm syringe filter (Corning). The remaining pellet was re-suspended using 5 ml of extraction solution before repeating the process and finally pooling the filtered supernatant. Dissolved microcystins samples were centrifuged (10 min, 3000 rpm) and supernatant was removed and filtered through a 0.2 mm syringe filter (Corning).

### **3.2.6.3 Microcystins Detection**

Microcystin-LR equivalents in blue crab tissues, particulate and feces samples, and dissolved microcystins samples were determined using a commercially available highly sensitive ( $0.04 \mu\text{g microcystins l}^{-1}$ ) enzyme-linked immunosorbent assay (ELISA) kit (Abraxis LLC), with a detection range of  $0.05\text{--}5.0 \mu\text{g microcystin-LR equivalents l}^{-1}$ . The indirect competitive assay allows for the congener-independent detection of microcystins, without cross reacting with non-related algal compounds. Samples were analyzed following the protocol included in the kit, with each sample run in duplicate and at several dilutions in order to reduce interference from matrix effects (impact of physical components of the sample on measurement of the analyte). For each

of the matrices tested, negative controls were run at the same dilutions as particulate, dissolved, and tissue samples to verify that matrix effects were minimized. Absorbance was read at 450 nm using a micro-plate spectrophotometer and all results are presented as  $\mu\text{g microcystin kg}^{-1} \text{ tissue}^{-1}$  (wet weight).

### **3.2.7 Lipid Hydroperoxide (LPO) Analysis**

#### **3.2.7.1 Lipid Hydroperoxide Extraction**

Lipid hydroperoxides were extracted into chloroform, according to protocol included in the lipid hydroperoxide assay kit from Cayman Chemical (USA). This protocol eliminates potential over-estimation due to hydrogen peroxides and ferric ions endogenous to the samples. Prior to extraction, hepatopancreas tissue samples were homogenized in 0.5 – 1.0 mL of HPLC-grade  $\text{H}_2\text{O}$ . A 0.5 mL aliquot of the homogenized tissue was placed into a glass test tube and 0.5 mL of deoxygenated methanol saturated Extract R was added before vortexing for 10 seconds. Cold deoxygenated chloroform (1.0 mL) was then added to the tube and vortexed for 10 seconds. The tubes were then centrifuged ( $1,500 \times g$ ,  $0^\circ\text{C}$ ) for 5 minutes before collecting the bottom chloroform layer. The extracts were stored frozen ( $-80^\circ\text{C}$ ) until the time of analysis.

#### **3.2.7.2 Lipid Hydroperoxide Detection**

Lipid hydroperoxides were quantified using a commercially available Lipid Hydroperoxide (LPO) Assay Kit and 96-well glass plate (Cayman Chemical, USA), with a sensitivity of 0.25 – 5 nmol hydroperoxide per assay tube. The assay uses redox reactions between hydroperoxides and ferrous ions, and the resulting ferric ions are measured spectrophotometrically using a thiocyanate ion chromagen. Following extraction, chloroform extracts were added to polypropylene microcentrifuge tubes, followed by deoxygenated chloroform-methanol solvent. Freshly prepared chromogen (0.05 mL) was added to the test tubes and vortexed for 10 seconds. The test tubes were held at room temperature for 5 minutes and 0.3



mL of the chromogen mixture was transferred to a glass 96-well plate. The absorbance was read at 500 nm using a micro-plate spectrophotometer and all results are presented at nmoles lipid hydroperoxide gram<sup>-1</sup> tissue<sup>-1</sup> (wet weight).

### **3.2.8 Statistical Analysis**

Two-way ANOVA was used for comparison of treatment dose versus microcystins accumulation in each tissue type and lipid hydroperoxide concentration. Three-way ANOVA was used for the factors: toxin congener, treatment dose, and tissue type versus data collected for microcystin accumulation or lipid hydroperoxide concentration. All pairwise comparisons were assessed using Tukey's test. Pearson's product moment correlation was used to assess the existence of correlation between total crab body weight loss and microcystins accumulation within the hepatopancreas. All statistical analysis was performed using Sigma Stat 3.1 statistical software package. Logarithmic transformations were applied in order to approach normality. For all statistical tests, significance level was set at  $P \leq 0.05$ , unless otherwise stated.

## **3.3 Results**

### **3.3.1 Preliminary Mortality and Behavioral Abnormality Observations**

In the crabs injected with either 1000 µg microcystin-LR kg<sup>-1</sup> crab body weight<sup>-1</sup> or 2000 µg microcystin-RR kg<sup>-1</sup> crab body weight<sup>-1</sup>, lethality was seen in the first but not in the latter within 24 hours following exposure. However, similar behavioral changes were seen in both crabs. Normal behavior, which included movement around tank, aggressive behavior towards objects nearby to tanks, and searching for food pieces in gravel, was apparent in both crabs within the first 5 hours following exposure. Approximately 5 hours following exposure, movement in both crabs slowed and they began to clean gills excessively. The crabs also appeared to have delayed or decreased aggression toward objects in or around the tanks.

### 3.3.2 Microcystin-LR and -RR 48-Hour Single-Exposure Experiment

The LD<sub>50</sub> of microcystin-LR and -RR in orally exposed blue crabs was >1000 µg kg<sup>-1</sup> of crab body weight. No lethality or behavioral abnormalities were seen in control or treatment groups during the 48-hour exposure period. Although low background toxicity was found in a few control (t=48) crabs, no significant (P>0.050) differences were seen in microcystin-LR or -RR accumulation between the control groups (t=0 and t=48). Accumulation of microcystin-LR and -RR occurred within the hepatopancreas and viscera of exposed blue crab (Fig. 3.2 A and B), with neither congener detected within the muscle or hypodermis tissues. The concentrations of microcystin-LR and -RR after the 48-hour exposure were not significantly different (P>0.050) in comparing between hepatopancreas and viscera tissue accumulation independent of dose, but were each found to be significantly (P<0.050) different than accumulation in muscle and hypodermis tissues. Accumulation of microcystin-LR in the viscera was found to be significantly different (P<0.050) between the doses of 10, 500, and 1000 µg microcystin-LR kg<sup>-1</sup> crab body weight<sup>-1</sup>, and these same doses were significantly different (P<0.050) than both controls. For microcystin-RR, accumulation within the viscera was found to be significantly different (P<0.050) between doses of 10 and 1000 µg kg<sup>-1</sup> crab body weight<sup>-1</sup>, and these same doses were significantly different (P<0.050) than both controls. Accumulation of microcystin-LR and -RR in the various tissues that were significantly different (P<0.050) than the controls (t=0 and t=48), are summarized in figures 3.2 A and B. At doses ≤500 µg kg<sup>-1</sup> crab body weight<sup>-1</sup>, highest mean toxin concentrations were found in the hepatopancreas. However, at a dose of 1000 µg kg<sup>-1</sup> crab body weight<sup>-1</sup>, higher mean toxin concentrations were found in the viscera tissues for both microcystin-LR and -RR (Fig. 3.2 A and B). There was no significant (P>0.050) dose-dependent accumulation of either microcystin-LR or -RR in any of the tissue types (hepatopancreas, viscera, muscle, or hypodermis) when microcystin concentrations in the tissues were analyzed

separately or together. However, when mean concentrations found in these tissues were summed for each treatment group, increased total accumulation was seen in response to an increased microcystin-LR or -RR dose (Fig. 3.3 A and B).

Total crab weight loss patterns (initial crab weight minus final crab weight) were positively correlated ( $P < 0.050$ ) with microcystin-LR accumulation within the hepatopancreas (Fig. 3.3A). However, crab weight loss and microcystin-RR concentrations found in the hepatopancreas were not as strongly correlated ( $P < 0.100$ ) as for microcystin-LR (Fig. 3.3B).

No microcystin-LR or -RR was detected in filtered particulate matter or in feces collected from aquariums. A low level ( $1.44 \mu\text{g l}^{-1}$ ) of dissolved microcystin was detected from water collected from a control ( $t=48$ ) treatment aquarium, from which the crab had a background toxicity of  $13.22 \mu\text{g microcystin kg}^{-1}$  hepatopancreas. No dissolved toxin concentrations were detected from aquarium water of crabs exposed to 10 or  $100 \mu\text{g microcystin-LR or -RR kg}^{-1}$  crab body weight<sup>-1</sup> doses. However, for some crabs exposed to doses  $\geq 500 \mu\text{g kg}^{-1}$  crab body weight<sup>-1</sup>, dissolved microcystins were detected in aquarium water. These concentrations ranged from below detection limit ( $0.10 \mu\text{g kg}^{-1}$ ) to  $2.59 \mu\text{g microcystin-LR l}^{-1}$  and below detection limit to  $7.40 \mu\text{g microcystin-RR l}^{-1}$ .

Lipid hydroperoxide levels in hepatopancreas tissues were not significantly ( $P > 0.050$ ) altered following 48-hour microcystin-LR or -RR exposure (Fig. 3.4 A and B). No significant ( $P > 0.050$ ) difference was seen in the lipid peroxidation between controls ( $t=0$  and  $t=48$ ).

In comparing between the two microcystin congeners, there was no significant difference ( $P > 0.05$ ) found in toxin accumulation, among the various tissues or between doses, or in lipid hydroperoxide concentrations (Fig. 3.5).

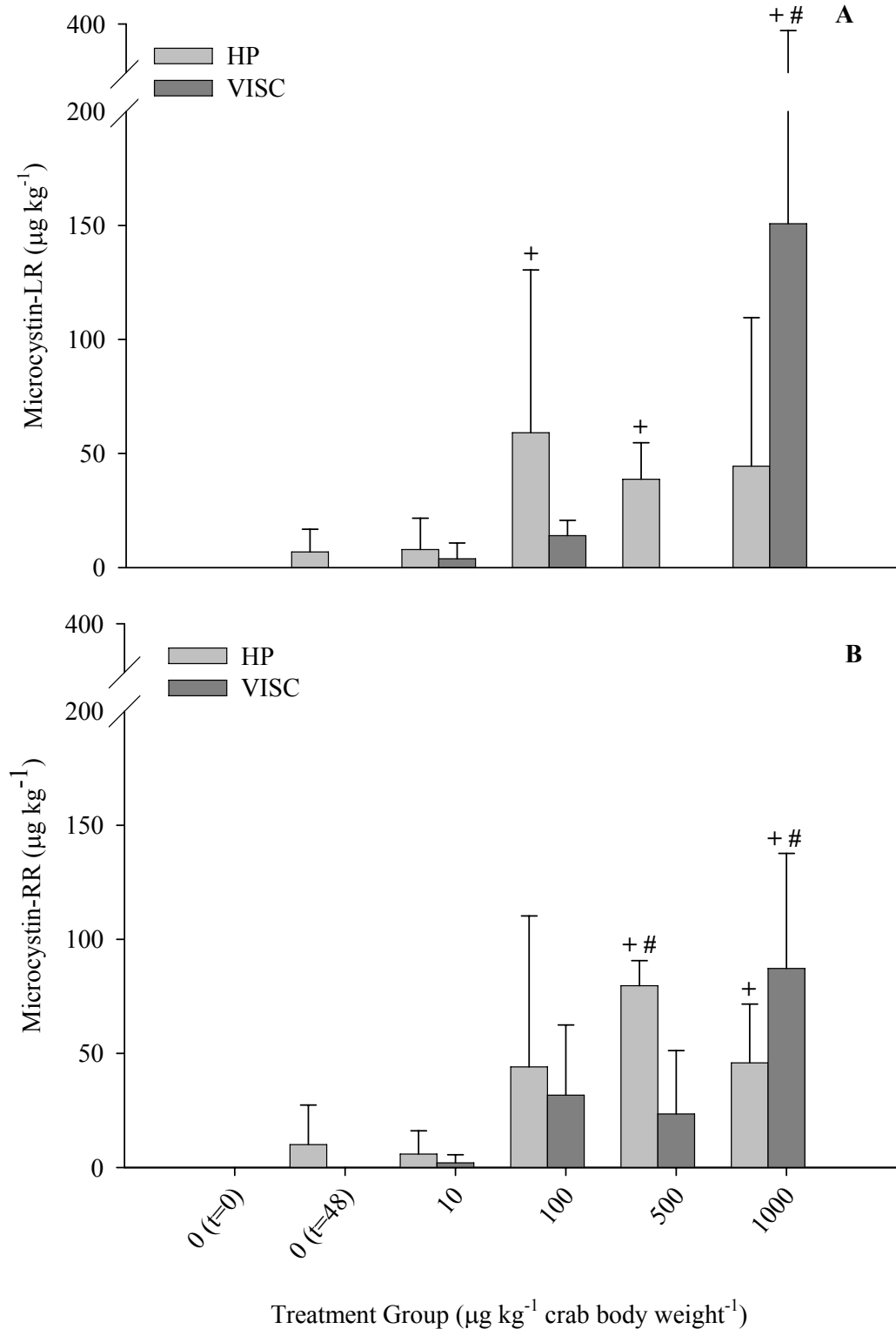


Figure 3.2. Accumulation of (a) microcystin-LR and (b) microcystin-RR in blue crab hepatopancreas (HP; light gray bar) and viscera (VISC; dark gray bar) following single oral treatment dose of either 0 (controls), 10, 100, 500, or 1000  $\mu\text{g kg}^{-1}$  crab body weight $^{-1}$  ( $n=3$ ). All data are expressed as means; + means significantly different ( $P<0.050$ ) from control ( $t=0$ ); # means significantly different ( $P<0.050$ ) from control ( $t=48$ ).

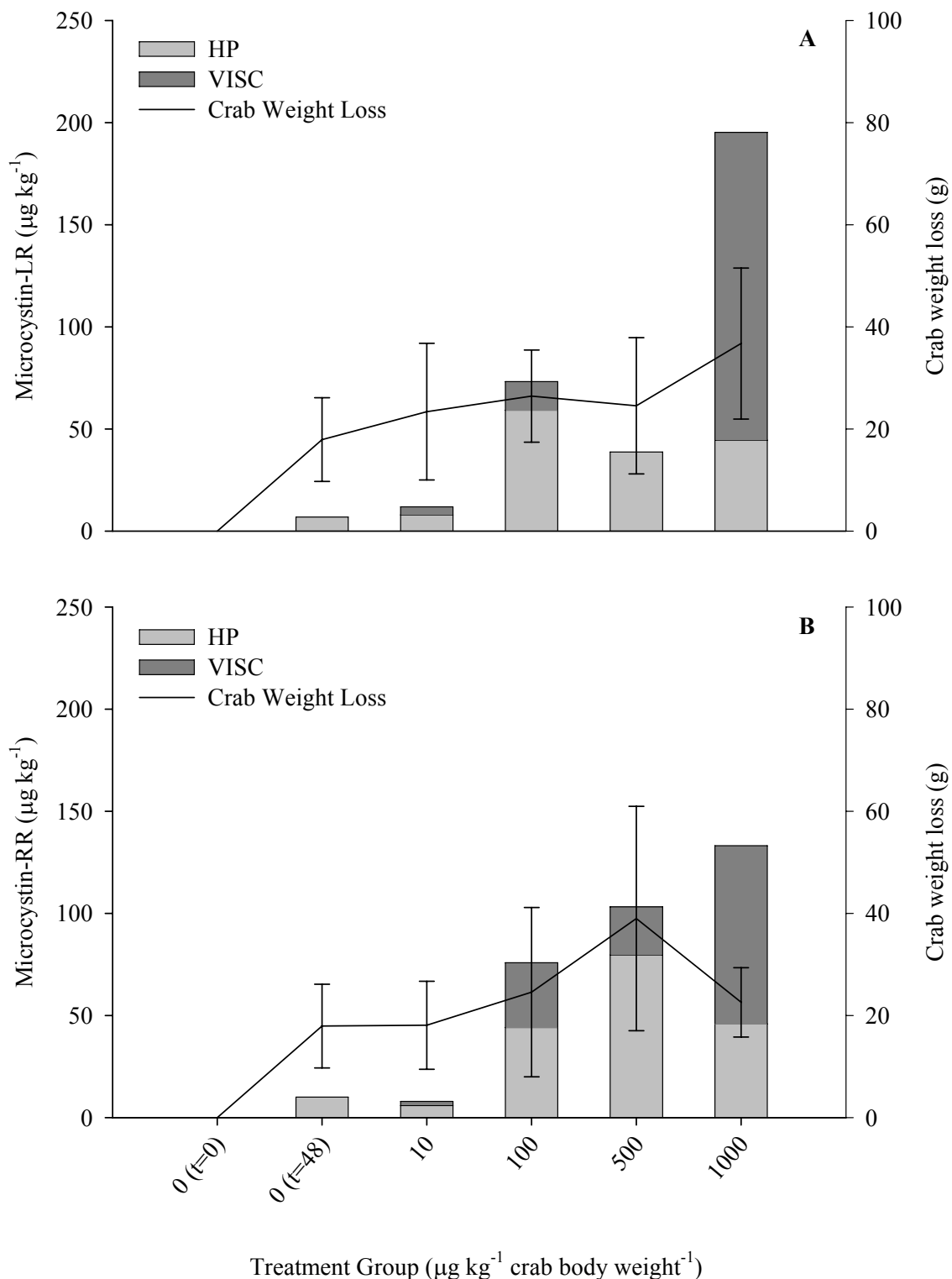


Figure 3.3. Total accumulation of (a) microcystin-LR and (b) microcystin-RR in blue crab hepatopancreas (HP; light gray bar) and viscera (VISC; dark gray bar) following single oral treatment dose of either 0 (controls), 10, 100, 500, or 1000  $\mu\text{g microcystin kg}^{-1}$  crab body weight $^{-1}$  ( $n=3$ ). Mean total crab weight loss (black line) was calculated as initial weight at start of experiment minus final weight 48 hours later for each treatment group.

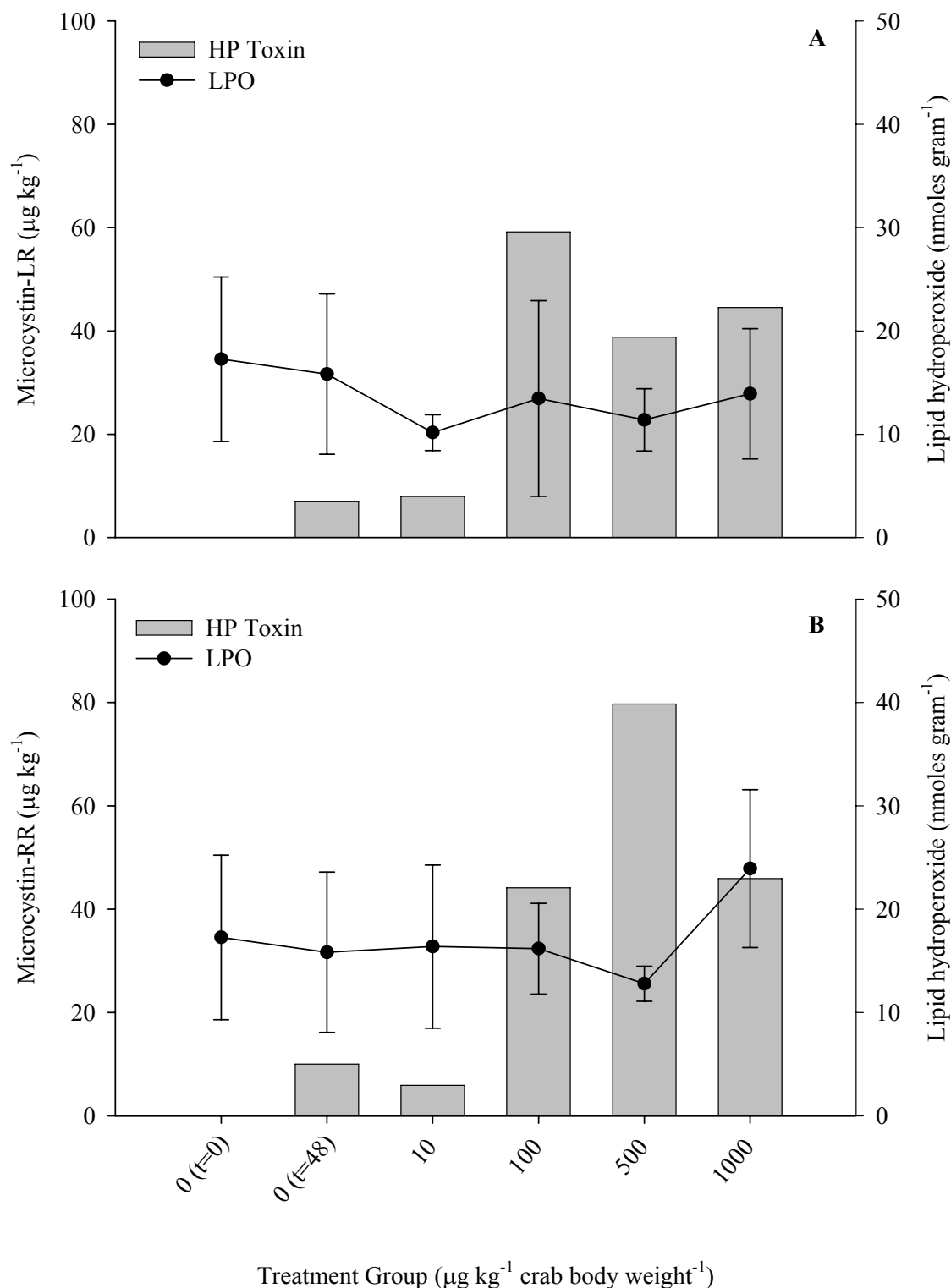


Figure 3.4. Lipid hydroperoxide concentrations (nmol gram $^{-1}$  wet tissue weight; black line) in hepatopancreas of blue crab following single oral treatment dose of either 0 (controls), 10, 100, 500, or 1000  $\mu\text{g kg}^{-1}$  crab body weight $^{-1}$  of (a) microcystin-LR and (b) microcystin-RR and corresponding accumulation of microcystin in blue crab hepatopancreas (HP toxin; light gray bar) ( $n=3$ ).

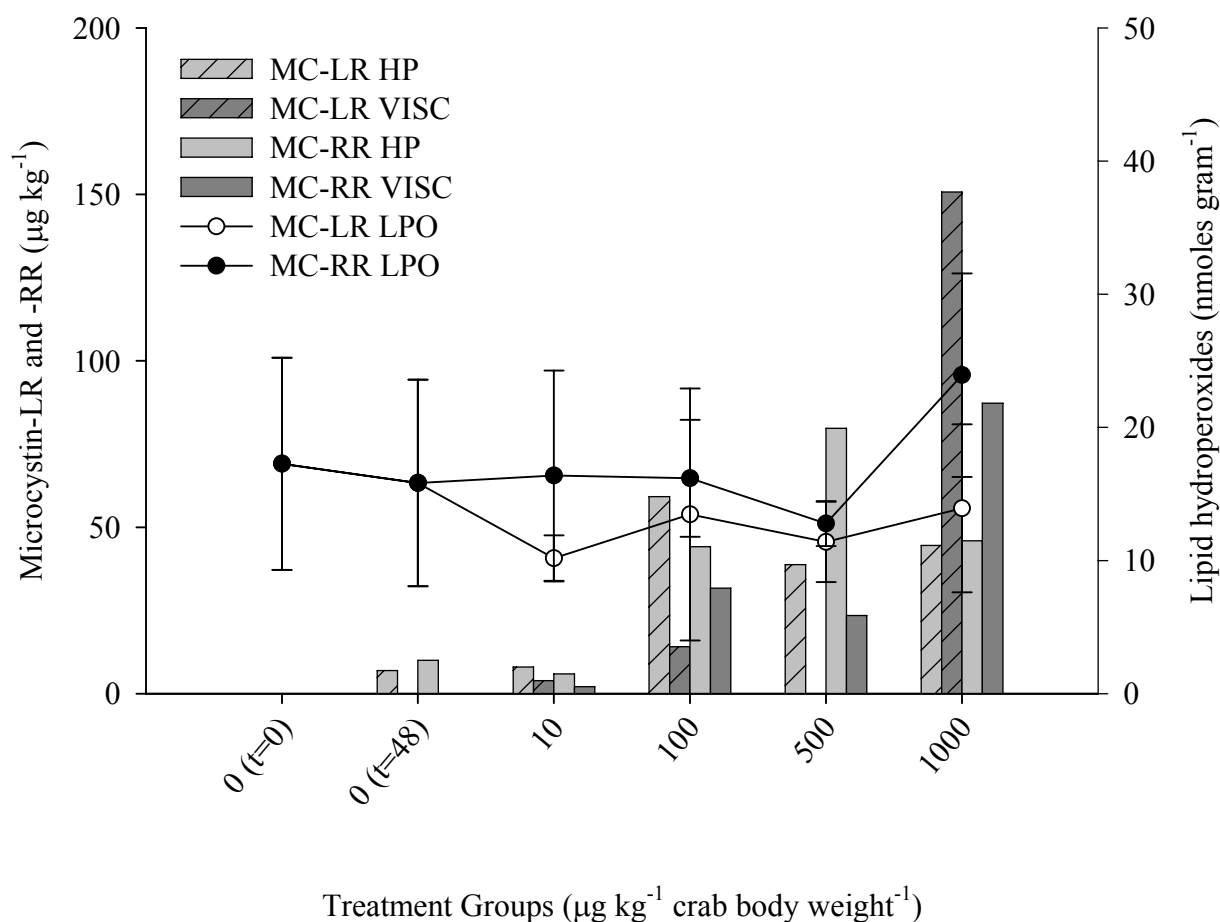


Figure 3.5. Accumulation of microcystins in hepatopancreas (light grey bars) and viscera (dark grey bars) and lipid hydroperoxide concentrations (nmol g<sup>-1</sup> wet tissue weight; black lines) in hepatopancreas of blue crab following single oral treatment dose of either 0 (controls), 10, 100, 500, or 1000 µg kg<sup>-1</sup> crab body weight<sup>-1</sup> of microcystin-LR (diagonally striped bars and filled circles) and microcystin-RR (plain bars and empty circles) (*n*=3).

### 3.3.3 Microcystin-RR Repeated-Exposure Experiment

No lethality or behavioral abnormalities were seen in either control or treatment groups during the 7-day (168-hour) microcystin-RR exposure period. There was no microcystin-RR detected from control crab tissues. Mean uptake of microcystin-RR occurred almost evenly in the hepatopancreas ( $115.08 \pm 139.48$  µg kg<sup>-1</sup>) and viscera ( $114.30 \pm 26.49$  µg kg<sup>-1</sup>) of the exposed blue crabs (Fig. 3.6), with no accumulation detected within the muscle, hypodermis, or gills.

Microcystin-RR accumulation within the hepatopancreas and viscera ranged from 5.29 to 304.05  $\mu\text{g kg}^{-1}$  and 83.93 to 141.85  $\mu\text{g kg}^{-1}$ , respectively. Toxin concentrations were significantly ( $P < 0.050$ ) increased after the 7-day (168-hour) exposure period compared to controls ( $t=168$ ) for both viscera and hepatopancreas tissue types. No microcystin-RR was detected in the filtered particulate matter, feces, or filtrate from aquarium water of control or treatment groups. Lipid hydroperoxide levels in hepatopancreas were significantly ( $P < 0.050$ ) augmented in crabs exposed to daily oral doses of 50  $\mu\text{g microcystin-RR kg}^{-1}$  crab body weight $^{-1}$  for 7 days ( $13.36 \pm 1.86$  nmoles gram $^{-1}$  wet tissue weight) in comparison to controls ( $t=168$ ) ( $7.14 \pm 3.93$  nmoles gram $^{-1}$  wet tissue weight) (Fig. 3.7).

### **3.4 Discussion**

The present study indicates that microcystin-LR and -RR were not acutely toxic to blue crab, since no mortality or behavioral abnormalities were seen despite significant accumulation of both congeners within hepatopancreas and viscera of blue crab 48 hours after consuming contaminated prey. The accumulation of either microcystin congener in the hepatopancreas and viscera was highly variable, especially at doses  $\geq 100 \mu\text{g kg}^{-1}$  crab body weight $^{-1}$ , likely resulting from natural physiological differences among the replicate crabs. Accumulation of both microcystin congeners was greatest in hepatopancreas at doses  $\leq 500 \mu\text{g kg}^{-1}$  crab body weight $^{-1}$ . However, at doses of 1000  $\mu\text{g microcystin-LR and -RR kg}^{-1}$  crab body weight $^{-1}$ , viscera tissue was shown to accumulate more toxin than hepatopancreas. This shift likely represents a series of physiological responses (i.e., depuration, decreased metabolism, and/or augmented detoxification reactions) to higher toxin doses, especially considering low levels of dissolved microcystins were also found in water from aquariums housing crabs receiving doses  $\geq 500 \mu\text{g kg}^{-1}$  crab body weight $^{-1}$ . Since aquariums were cleaned at the start of each experiment, these dissolved toxins either represent depuration during the 48-hour exposure period by the crabs or leakage of toxin



from fish during feeding. However, the concentrations of these dissolved microcystins were too low to singly account for this shift. A similar shift was indicated by Dewes et al. (2006), who measured highest mean accumulation in hepatopancreas of *Chasmagnathus granulatus* of  $32.14 \pm 4.12 \mu\text{g kg}^{-1}$  after exposure to  $172 \mu\text{g microcystins kg}^{-1}$ . In the same study, crabs were exposed to  $860 \mu\text{g kg}^{-1}$ , but only accumulated  $13.17 \pm 0.56 \mu\text{g microcystins kg}^{-1}$  within the hepatopancreas.

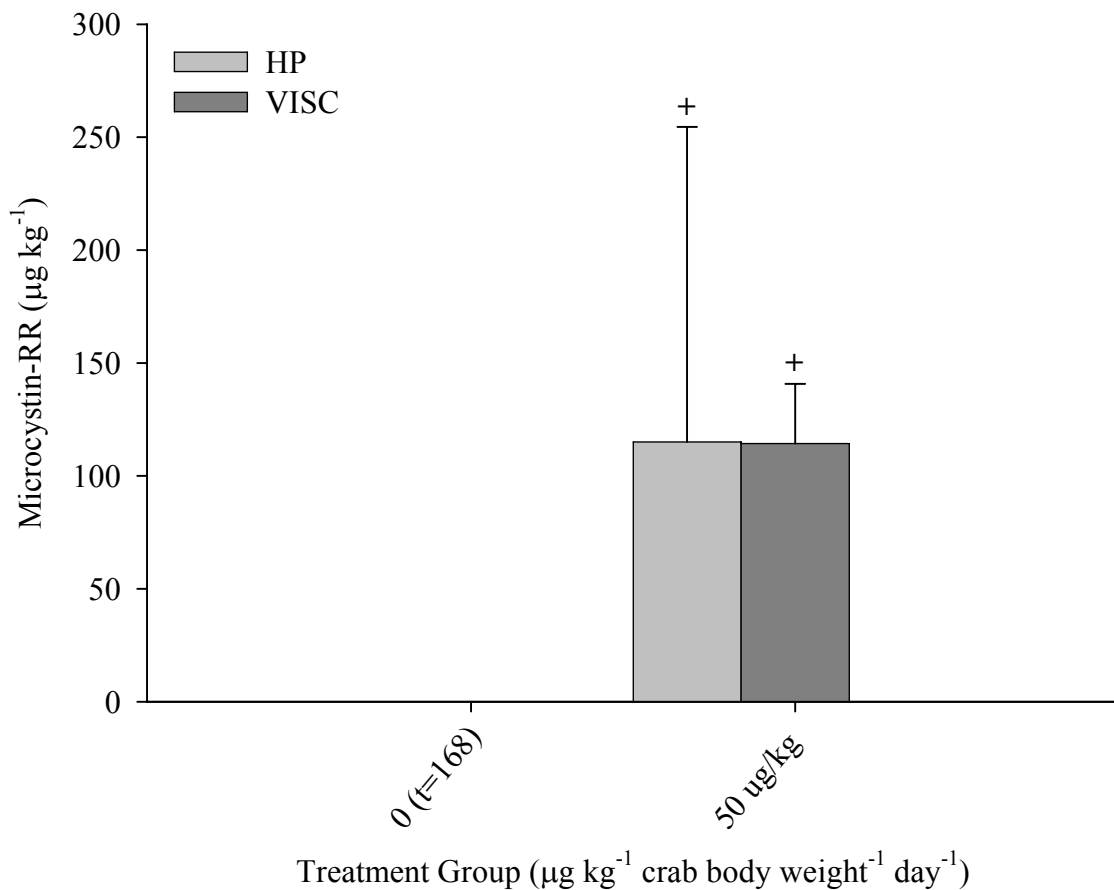


Figure 3.6. Accumulation of microcystin-RR in blue crab hepatopancreas (HP; light gray bar) and viscera (VISC; dark gray bar) after 7 days (168 hours) of daily oral treatment doses of either 0 (control (t=168)) or  $50 \mu\text{g microcystin-RR kg}^{-1}$  crab body weight $^{-1}$  day $^{-1}$  ( $n=3$  and  $n=4$ , respectively). Microcystin-RR concentration in muscles, hypodermis, and gills were below detection limits for all samples (not shown). All data are expressed as means; + means significantly different ( $P<0.050$ ) from control (t=168-hrs.).

In the 7-day (168-hour) repeated dose experiment of the present study, mean accumulation was nearly equal within hepatopancreas and viscera. However, when accumulation values for hepatopancreas and viscera were examined separately for each replicate, this trend was no longer evident, and a high accumulation in one tissue was usually matched by a low accumulation in the other. Low or no depuration occurred during the 7-day (168-hour) experiment, as made evident by a lack of detectable particulate or dissolved microcystins in aquarium water or feces.

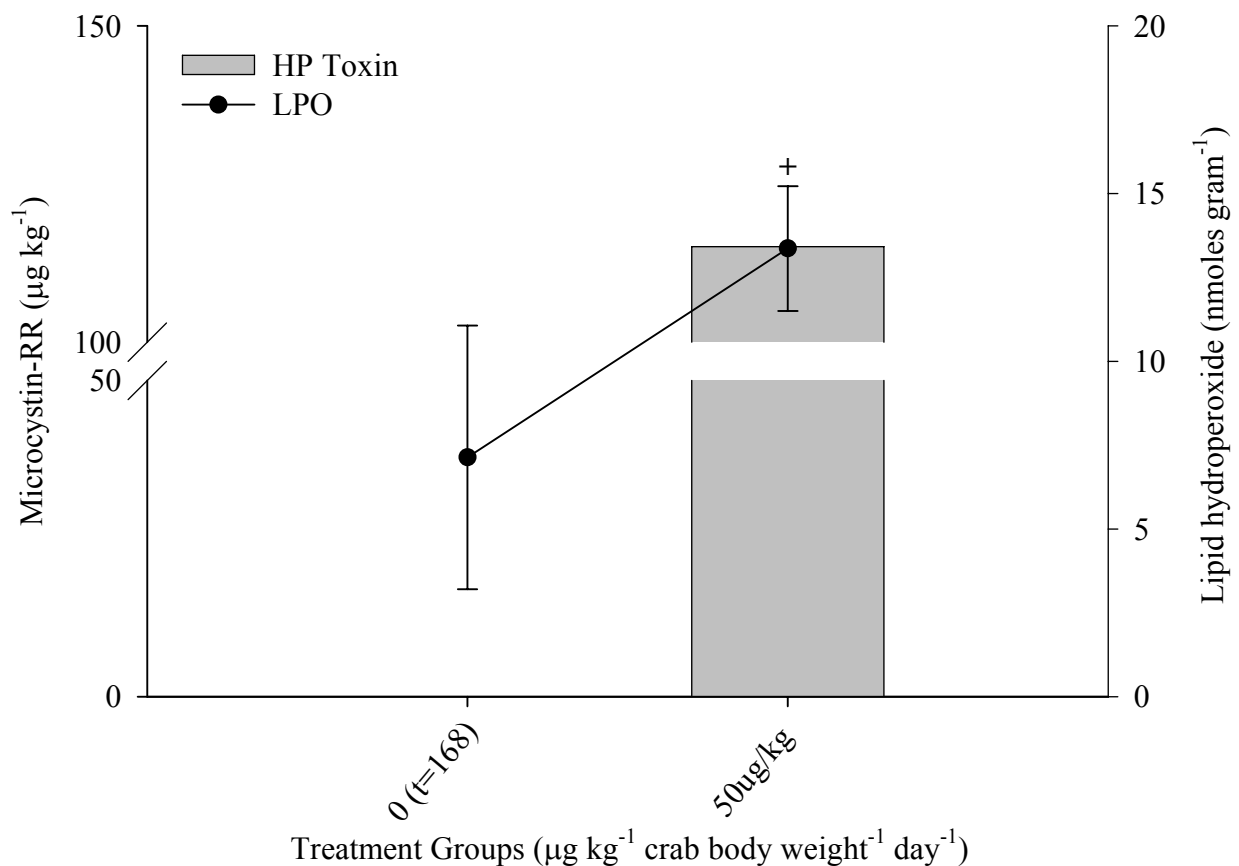


Figure 3.7. Lipid hydroperoxide concentrations (nmoles gram<sup>-1</sup> hepatopancreas<sup>-1</sup>; black line) in hepatopancreas of blue crab after 7 days (168 hrs) of daily oral treatment doses of either 0 (controls (t=168)) or 50 µg microcystin-RR kg<sup>-1</sup> crab body weight<sup>-1</sup> day<sup>-1</sup> (n=3 and n=4, respectively) and corresponding accumulation of microcystin-RR in blue crab hepatopancreas (HP toxin; light gray bar). All data are expressed as means; + means significantly different (P<0.050) from control (t=168).

Depuration likely occurred during the 48-hour experiment from crabs previously mentioned and from a single control (t=48) crab, where  $1.44 \mu\text{g}$  dissolved microcystin  $\text{l}^{-1}$  was detected from the aquarium water. This control crab had background toxicity likely derived from a previous natural exposure, of  $13.22 \mu\text{g}$  microcystin  $\text{kg}^{-1}$  hepatopancreas. Out of the twelve control crabs used in the 48-hour experiments, 25% had background hepatopancreas toxicity and these crabs were collected from a single date in October 2009.

Despite significant accumulation within hepatopancreas and viscera in both the shorter-term and longer-term experiments, neither exposure period resulted in accumulation of either microcystin-LR or -RR within muscle. This is likely due to insufficient time for absorption of microcystins from the digestive tract. Results from studies on cichlids, rainbow trout, and carp report that surface area and pH of fish digestive tracts influence microcystin absorption capacities (Ibelings and Havens, 2007 and references therein). Although, efficient microcystin breakdown during digestion could also decrease these concentrations in muscle tissues to concentrations below detection limits of ELISA. Lower instances of microcystin accumulation in muscle tissue of natural populations of blue crab were also demonstrated in a study by Garcia et al. (2010), where only 37.5% of muscle tissues measured had detectable concentrations compared to high instances of accumulation in hepatopancreas and viscera, 77.8% and 94.1%, respectively.

No accumulation of microcystins in hypodermis or gills of blue crabs was observed during the present study. This may indicate that these are not significant tissues of accumulation and depuration of microcystins in blue crabs, however, data from the present study were not sufficient to draw conclusions.

No significant difference was seen between accumulation of the two congeners, microcystin-LR and -RR, as previously suggested by Xie et al. (2004). However, this difference may have become evident if microcystin accumulation in muscle tissue had occurred.

A positive correlation was seen between total crab weight loss and microcystin-LR accumulation in the hepatopancreas after the 48-hour exposure period. Although crab weight loss and microcystin-RR were not as strongly correlated ( $P=0.069$ ) likely due to greater variation, the same general pattern could be seen. Highest weight loss occurred at doses  $\geq 500 \mu\text{g kg}^{-1}$  crab body weight<sup>-1</sup> of either microcystin congener. This finding is consistent with a study by Jang et al. (2004), which found that 98% of fish fed with toxic *Microcystis aeruginosa* lost weight, while fish fed with *Scenedesmus* (green algae) or a commercial pellet food gained weight. Lance et al. (2008) found that *Potamopyrus antipodarium* (Gastropoda) that fed on the microcystin-producing cyanobacteria, *Planktothrix agardhii*, experienced decreased growth and fecundity. Changes found in blue crab are likely the result of trade-offs at times of high metabolic cost (see also Marinovic and Mangel, 1999) between resources/energy necessary for growth and those necessary for detoxification/antioxidant mechanisms (Lance et al., 2008; Cazenave et al., 2006).

Multiple oxidative reactions occur within the crustacean hepatopancreas, which may be the main site of reactive oxygen species (ROS) generation (Pinho et al., 2003). However, single doses up to  $1000 \mu\text{g kg}^{-1}$  crab body weight<sup>-1</sup> microcystin-LR or -RR were not sufficient to significantly alter lipid peroxidation within blue crab hepatopancreas despite high internal accumulation of both microcystins. The results indicate that oxidative damage to lipids was averted possibly due to augmented antioxidant defenses in response to microcystin exposure. Dewes et al. (2006) showed augmented Glutathione *S*-transferase (GST) activity, but no changes to lipid peroxidation, in hepatopancreas of the crab, *Chasmagnathus granulatus*, after short-term exposures of 6 and 12 hours to  $860 \mu\text{g kg}^{-1}$  of microcystins. However, in the 7-day (168-hour)

exposure experiment of the present study, daily low-level doses of 50 µg microcystin-RR kg<sup>-1</sup> crab body weight<sup>-1</sup> day<sup>-1</sup> induced oxidative stress, significantly increasing lipid hydroperoxides in crab hepatopancreas. High variation in lipid peroxidation found from both the 48-hour and 7-day (168-hour) experiments, may have captured physiological variability associated with antioxidant responses and the accumulation of microcystins among the individual crab replicates. These results may have significant implications for monitoring of microcystins in commercially important areas, as repeated low-level exposure may impact organism health more severely than short-term high-level microcystin exposure.

Vasconcelos et al. (2001) showed that compounds, other than microcystins, are present in cyanobacterial cells and these may have detrimental impacts on aquatic invertebrate populations. In the present study, purified extracts of microcystin-LR and -RR from *Microcystis aeruginosa* were used. Therefore, impacts seen in the present study could significantly increase if blue crabs are exposed naturally to whole cells of microcystin-producing cyanobacteria (i.e., from the gut contents of filter-feeding prey such as *Rangia cuneata*). Additionally, the methanol extraction method for tissue samples used in this study will not extract covalently bound microcystins. Thus, accumulation of microcystins in tissue samples of this study are underestimated, although, the toxicity of these bound microcystins is largely unknown (Ibelings et al., 2005). Further toxicity characterization of whole cyanobacterial cells and covalently bound microcystins, is needed to better understand the potential impacts of increasing cyanobacterial blooms on blue crab health.

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## CHAPTER 4

### HEPATOTOXIC CYANOBACTERIAL BLOOMS IN LOUISIANA'S ESTUARIES: ANALYSIS OF RISK TO BLUE CRAB (*CALLINECTES SAPIDUS*) FOLLOWING EXPOSURE TO MICROCYSTINS: CONCLUSIONS

The studies presented in the previous chapters were aimed at assessing the potential risk of increasing blooms of toxic cyanobacteria, specifically microcystin-producers such as *Microcystis* and *Anabaena* spp., in eutrophic estuaries of Louisiana and to further understand how the key-predator, blue crab and associated fisheries are affected by these potent hepatotoxins. The field study (Chapter 2) was conducted in a hyper-eutrophic freshwater lake, Lac des Allemands, located in the Barataria Estuary System of southeastern Louisiana, and documented cyanobacterial abundances and microcystin concentrations in surface water and blue crab tissues. The laboratory study (Chapter 3) documented the distribution and physiological effects of microcystin-LR and -RR in orally exposed blue crabs.

In chapter 1, I introduced two major estuaries in Louisiana, Barataria-Terrebonne Estuary System and Lake Pontchartrain. These are highly productive systems with large commercial fisheries of blue crab, contributing significantly to the total dockside value of blue crab in Louisiana of approximately \$44.8 million in 2007 (LDWF, 2008). However, accelerated nutrient enrichment of the Barataria-Terrebonne Estuary System may be increasing microcystin concentrations in surface waters and in aquatic food webs.

In chapter 2, results confirmed that toxic *Anabaena* and *Microcystis* spp. abundances were sufficient to potentially contaminate intermediate filter-feeding bivalves, such as clams and mussels in the upper Barataria estuary. Multiple organs of the blue crab, *Callinectes sapidus*, harvested during the commercial and recreational harvest season were shown to be contaminated with microcystins, most likely as a result of consuming contaminated prey, such as bivalves and chironomids (Galván et al., unpublished data). Given the trajectory of nutrient increases, the

presence of cyanobacterial toxins in water and organisms from the hyper-eutrophic Lac des Allemands demonstrates the need to continuously monitor water for harmful cyanobacterial blooms, concentrations of cyanobacterial toxins, and accumulation of cyanobacterial toxins in components of the food web.

In chapter 3, significant accumulation of microcystin-LR and -RR congeners was found in hepatopancreas and viscera tissues of blue crab following a single exposure to various moderate and high doses, however, no oxidative stress was induced in the hepatopancreas after 48 hours. These results indicated decreased crab weight was positively correlated with increased accumulation of microcystin-LR and -RR in the hepatopancreas. These results could have important consequences for commercial fisheries of blue crab, especially if fecundity (not measured in this study) and ecdysis are also affected. Following daily exposure to concentrations equivalent to or less than those previously found in *Rangia cuneata* (Galván et al., unpublished data), a common prey item of blue crab, lipid peroxidation increased indicating that antioxidant defenses were overwhelmed, and therefore oxidative stress occurred. Thus, low-level repeated exposure to a less toxic microcystin congener (relative to microcystin-LR) may have significant impacts on blue crab health.

The aforementioned studies highlight the vulnerability of Louisiana's estuaries and fisheries to nutrient enrichment processes that promote toxic and noxious phytoplankton blooms. Similar feeding strategies employed by catfish and crayfish indicate that cyanotoxin contamination may occur within additional fisheries that are commercially important to Louisiana. For example, this study has already documented microcystin concentrations within multiple blue catfish, *Ictalurus furcatus*, taken from Lac des Allemands (Chapter 2). Vasconcelos et al. (2001) have also documented microcystin uptake in crawfish, *Procambarus clarkii*, following exposure in laboratory experiments. Blue catfish and crawfish ranked

thirteenth and seventh, respectively, among all commercially harvested seafood species in Louisiana in 2002 by dockside value (LDWF, 2004). However, this study is also applicable throughout the United States, wherever Louisiana blue crabs are consumed or where similar cyanobacterial blooms are occurring.

Further investigation of physiological changes induced by the various microcystin congeners, such as lipid peroxidation (LPO), DNA damage, mitochondrial damage, stimulation of enzyme and antioxidant activities (e.g., Glutathione *S*-transferase (GST), Tripeptide Glutathione (GSH), Superoxide Dismutase (SOD), and Catalase (CAT)), oxygen consumption, and hepato-histological damage (Amado and Monserrat, 2010 and references therein, Pinho et al., 2005), should be investigated in blue crabs and relevant aquatic organisms in order to further determine risk of toxic cyanobacteria on organism health and fishery production. However, further assessment of trophic transfer of microcystins from these commercially important fisheries to higher order consumers, including humans, is also needed as these health hazard studies may further promote legislation aimed at control of excess nutrients leading to eutrophication and associated noxious and toxic harmful algal blooms (Codd, 2000).

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## **VITA**

Ana Cristina Garcia was born in Atlanta, Georgia, in June 1984, and is the daughter of a pharmacist (Mildred R. Garcia) and an architect (Rafael A. Garcia). She is the sister of Rafi Garcia, an underwater film producer interested in Mayan archeology of the Yucatán. In 2002, Ana began her undergraduate career at Louisiana State University, culminating in a Bachelor of Arts degree in animal sciences in May 2006. She is now a candidate for a Master of Science degree in the Department of Oceanography and Coastal Sciences from Louisiana State University in May of 2010.